Volume 50 MARCH, 1952 Number 3

ENDOCRINOLOGY

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MORRIS, DAVID M.
The Thyrotrophic Activity of the Pituitary of the White Leghorn Cockerel as Related to its Cytology 277

MARCUS, FRANK, LOUISE P. ROMANOFF AND GREGORY PINCUS
The Electrolyte-Excreting Activity of Adrenocortical Substances 286

MCSHAN, W. H., AND ROLAND K. MEYER
Gonadotrophic Activity of Granule Fractions Obtained from Anterior Pituitary Glands of Castrate Rats 294

MCARTHUR, JANET W.
The Identification of Pituitary Interstitial Cell Stimulating Hormone in Human Urine 304

LEATHEM, JAMES H., AND LESLIE A. STAUBER
Adrenal Lipid and Alkaline Phosphatase in Hamsters Infected with Leishmania Donovani 311

MAYER, JEAN, RUTH E. RUSSELL, MARGARET W. BATES, AND MARGARET M. DICKIE
Basal Oxygen Consumption of Hereditarily Obese and Diabetic Mice 318

ALBERT, A., ALIDA TENNEY AND ELIZABETH FORD
The Effect of Cortisone and Corticotropin on the Biologic Decay of Thyroidal Radioiodine 324

ALBERT, A., ALIDA TENNEY AND NONA LORENZ
The Effect of Hypophysectomy on the Renal Clearance of I^{131} 327

GROLLMAN, ARTHUR
The Maintenance of the Adrenalectomized Dog for Prolonged Periods Without Recourse to Hormonal Therapy 331

SELIGMAN, ARNOLD M. AND RIVKA ASHBEL
Histochemical Demonstration of Ketosteroids in Adrenal Cortical Tumors with or without an Associated Cushing's Syndrome 338

AMES, ROSE G. AND H. B. VAN DYKE
Antidiuretic Hormone in the Serum or Plasma of Rats 350

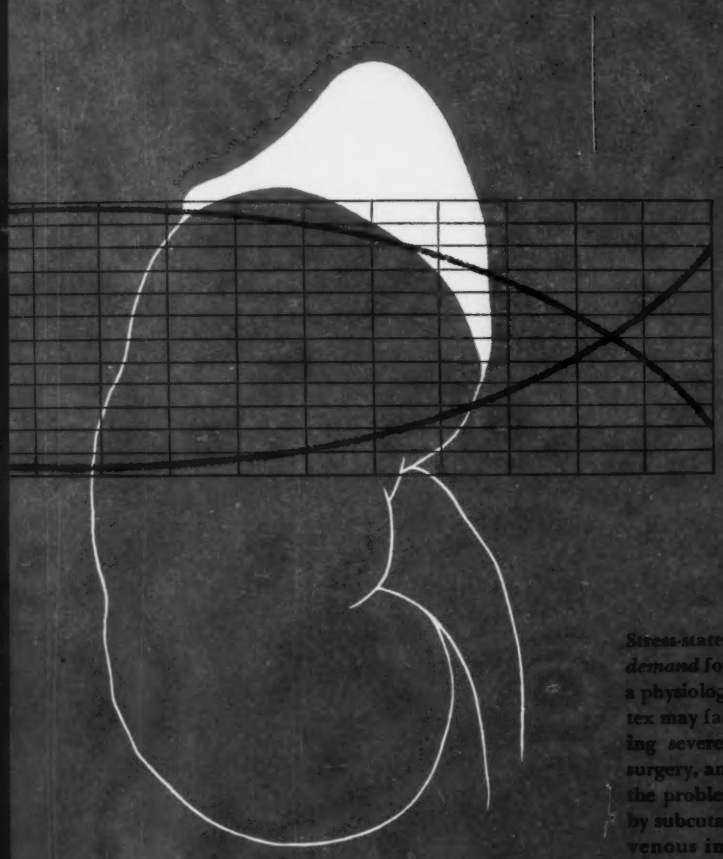
KALMAN, SUMNER M.
The Effect of Androgens on the Succinoxidase System of Rat Liver 361

SAVARD, KENNETH, WILLEM J. KOLFF AND ARTHUR C. CORCORAN
Corticosteroids of Peripheral Blood 366

NOTES AND COMMENTS
ALBERT, A., ALIDA TENNEY AND NONA LORENZ
The Absorption of Thyroxine from the Gastrointestinal Tract of the Rat. 374

ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY
BY CHARLES C THOMAS, PUBLISHER,
SPRINGFIELD, ILLINOIS

Annual Subscription $12.00 Single Copies $1.50



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ENDOCRINOLOGY

VOLUME 50 MARCH, 1951 NUMBER 3

THE THYROTROPHIC ACTIVITY OF THE PITUITARY OF THE WHITE LEGHORN COCKEREL AS RELATED TO ITS CYTOLOGY*

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INTRODUCTION

IT WAS recognized as early as 1898 by Comte that a relationship exists between the cytological picture of the pituitary and the physiological state of the animal. Numerous attempts have been made to correlate the function of the anterior hypophysis with its cytology, but it cannot yet be said with certainty that a particular pituitary cell type is responsible for the elaboration of a certain hormone. Perhaps the closest approach to unanimity of opinion in this matter is in regard to the origin of the follicle stimulating hormone from basophiles. This is supported by the cytological work of Severinghaus with the rat (1937), Payne with the chick (1947), and others, and by such experimental work as that of Schooley and Riddle on the pigeon (1938) and Breneman on the chick (1941).

The origin of the luteinizing hormone from the acidophiles is a little less certain, while considerable confusion exists regarding the cytological source of the thyrotrophic hormone. On the basis of cytological studies of the rat hypophysis, Severinghaus (1937, 1939) concluded that the acidophiles secrete the thyrotrophic hormone while Zwecker *et al.* (1935) described the vacuolation of what they believed to be basophiles, after thyroidectomy and suggested that these "thyroidectomy cells" secrete the thyrotrophic hormone. Payne (1944) also described "thyroidectomy

Received for publication November 25, 1949.

* Presented to the faculty of the graduate school, Indiana University, in partial fulfillment of the requirements for the Doctor of Philosophy degree in Zoology.

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cells" in the pituitary of the chick after removal of the thyroid and agreed that they might be the source of the thyroid stimulating principle but did not confirm Zwecker's theory as to their origin. Payne believes the thyroidectomy cells originate "for the most part, if not in all cases, from chromophobes."

A very detailed study by Payne (1942) of the cytological changes of the pituitary of the White Leghorn fowl subjected to various treatments, suggested an approach to the problem of cell source of thyrotrophin. Thus, for example, Payne found that pituitaries of birds which had been kept on a quantitatively restricted diet were largely acidophilic in nature while those of castrate birds were predominantly basophilic. Other treatments yielded pituitaries variously acidophilic or basophilic in character. In the present study some of the treatments used by Payne were duplicated as nearly as possible to obtain birds with pituitaries of known cytology. The thyroids of the treated animals were studied and the pituitaries assayed for thyrotrophic activity in an attempt to correlate activity with pituitary cytology.

MATERIALS AND METHODS

Treatments included limited diet, castration, estrogen injection, estrogen injection with limited diet, androgen injection, and thiouracil feeding. The thyrotrophic activity of the pituitaries was determined by analysis of weights and follicular cell heights of the thyroids of treated animals and of assay chicks.

For histological purposes paraffin serial sections of thyroids fixed in 10 per cent formalin were made at 5 microns and stained with Heller's modification of Delafield's Hematoxylin and with Eosin. Measurements of follicular cells were made under oil immersion with an ocular micrometer.[3] Usually five, but occasionally four, thyroids of each series were "read" by measuring the height of fifty follicular cells in each gland. An effort was made to obtain a representative sample by measuring only one or two cells per field, starting at one edge of a section, crossing that section once, and moving to the next section.

The pituitaries of a given series were weighed en masse and dried in acetone. At the time of assay they were removed from the acetone and dried under a desk lamp. Their dry weights were determined, and then they were ground in a mortar and taken up in a measured amount of distilled water. Two-tenths milliliters of the suspension was injected into day-old chicks on the first evening of their arrival and similar injections were made in the morning and evening of the second and third days. Doses were calculated so that each chick received 5 milligrams, equivalent wet weight, of pituitary tissue over the entire period. A series of control chicks, receiving a similar course of injections of distilled water, was run with each assay. The animals were sacrificed about 12 hours after the last injection, and their thyroids were weighed and fixed in 10% formalin for histological study. Chicks were kept in their shipping boxes without feed during the assay procedure.

[3] Follicular cell height measurements are recorded in units. With the combination of lenses used, 1 unit was equivalent to approximately 1.4 microns.

Limited diet was determined as an amount of chicken mash equivalent to half that consumed by the control animals the preceding day. The controls had food before them at all times and experimental animals were fed the daily ration each morning all at once. This method of treatment was somewhat different from that used by Payne whose cytological studies of pituitaries are used in the analysis of the data presented here. Payne removed the food from his experimental birds on alternate days. The present method permitted better control of the diet, and it is felt that the physiological effects are comparable to those obtained in Payne's work.

A 0.1% thiouracil feed was prepared by mixing an appropriate amount of thiouracil powder[4] with regular chicken mash. This was fed ad libitum.

RESULTS AND DISCUSSION

Limited diet

Several series of birds of various ages were subjected to various periods of limited diet as shown in table 1. The thyrotrophic activity of the pituitary as judged by relative thyroid weights was not altered appreciably by this treatment in the younger birds but in the older birds the effect of the restricted diet over comparable periods of time was reflected in a decreased thyroid weight. The relative pituitary weight was not significantly altered by the experimental diet although the absolute values were lower in all the experimental series tested. Payne (1942) had also observed that pituitaries of 15- to 30-day-old birds kept on a limited diet were smaller than those of control birds of the same age. This suggests a decrease in total activity of the pituitary of the limited-diet birds. The thyrotrophic potency of such pituitaries as assayed with the day-old chick was indeed significantly less in the older series but not in the younger series.

According to Payne the pituitary of limited diet birds from 15 to 31 days of age shows fewer and less active basophiles while the number of acidophiles remains unchanged or is perhaps slightly increased. It is probably justifiable to project this pituitary picture into the present older series, at least with regard to the basophiles, since gonad weights are lower in older limited-diet birds as well as in the younger ones observed by Payne and Breneman (Morris, 1951). Correlating this cytological picture with the potency data one is tempted to ascribe thyrotrophic activity to the basophiles, although data on the nature and number of the acidophiles in the older limited-diet birds is not known. This hypothesis was tested by studying the activity of strongly basophilic pituitaries.

Castration

Payne (1942) reported definite changes in pituitaries of chicks castrated at varying times prior to 7 days of age. These changes are principally an

[4] Thiouracil powder was supplied through the courtesy of the Lederle laboratories.

TABLE 1. EFFECT OF LIMITED DIET ON THYROIDS AND PITUITARIES

Treatment	No.	Body gms.	Thyroid		Pituitary wt.*	Pituitary assay	
			Wt.*	Cell ht.		No.	Thyroid wt. (mg.)
Treatment begun at 4 days of age							
LD 4 days	15	47	7.37	0.94	1.68	6	5.50
ND	10	55	7.45	1.03	1.95	5	5.06
LD 11 days	12	65	8.80		1.70	3	3.87
ND	12	82	7.60		1.80	5	4.14
LD 25 days	21	108	6.67	2.00	1.74	13	4.60
ND	20	218	6.29	1.65	1.49	9	4.25
Treatment begun at 10 days of age							
LD 2–12 hrs§	26	48	6.77				
ND	30	58	6.31				
LD 1 day	3	59	5.59				
ND	3	60	4.34				
LD 2 days	3	53	7.78				
ND	3	63	6.83				
LD 3 days	3	63	5.87				
ND	3	79	6.33				
LD 4 days	3	67	4.03				
ND	3	79	4.69				
LD 5 days	3	57	5.97				
ND	3	82	6.10				
LD 6 days	3	61	5.90				
ND	3	84	5.45				
LD 7 days	5	62	5.33				
ND	9	87	4.72				
LD 10 days	8	65	5.70	1.69	1.38		
ND	9	101	7.92	2.33	1.68		
Treatment begun at 58 days of age							
LD 7 days	18	596	7.60		1.20	9	5.02
ND	18	678	8.10		1.12	5	4.92
LD 14 days	17	652	6.50‡		1.09	8	5.32
ND	17	840	8.50		1.03	8	5.44
LD 28 days	15	786	7.30		1.36	8	4.02‡
ND	19	1082	7.70		1.73	10	5.79
LD 42 days	18	871	7.40†		0.74	9	4.15‡
ND	18	1300	8.70		0.65	6	5.70
Assay control, water injected						18	4.40

* Weights recorded as mgs. per 100 gms. body weight.
†,‡ Difference from respective control significant at the 5% and 1% levels respectively.
§ Figures represent mean values from birds autopsied at two-hour intervals for the first twelve hours.
LD = Limited diet; ND = Normal diet.

increase both in size and number of the basophiles and an increase in the size of the nucleoli of the basophiles. He also reported a degranulation and regression in number of the acidophiles attendant upon castration.

Despite this radically altered pituitary present in the castrated bird,

TABLE 2. EFFECT OF VARIOUS TREATMENTS ON THYROIDS AND PITUITARIES

Treatment, age at autopsy	No.	Body gms.	Thyroids		Pituitary wt.*	Pituitary assay (thyroid cell ht.)
			Wt.*	Cell ht.		
Capon 43 days old	11	440	5.65	2.15	1.39	1.34
Control 43 days old	14	433	5.11	2.30	1.02	1.69
Capon 57 days old	9	659	5.84	2.78†	1.36	1.42
Control 57 days old	14	707	5.43	3.28	0.91	1.34
Capon 80 days old	10	1133	6.10	—	—	3.48§
Control 80 days old	6	1203	6.65	—	—	3.52§
Estrone 20 days old	18	112	10.20	3.02†	1.97	1.40
Control 20 days old	10	108	5.80	2.47	1.82	1.13
Estr., LD 20 days old	16	92	7.70	2.26	2.44	1.41
LD 20 days old	5	86	7.80	2.54	2.01	1.18
Androgen 31 days old	19	250	5.50	2.82	1.64	1.99
Control 31 days old	10	241	7.30	3.26	1.69	2.57
Androgen 46 days old	13	476	3.74	2.38	0.93	2.24
Control 46 days old	8	421	4.03	2.19	0.98	1.20
Thiouracil 25 days old	18	140	48.20‡	4.63‡	2.39	1.19
Control 25 days old	11	173	5.70	2.53	1.66	2.43

* Thyroid and pituitary weights are expressed as mgs. per 100 gms. body weight.
†,‡ Difference from control significant at the 5% and 1% level respectively.
§ These figures represent mean weight of thyroid glands in milligrams. Follicular cell heights were not determined for these thyroids.

little or no change was observed in the thyrotrophic activity (Table 2). The weight changes in the pituitary agree with those of Payne, showing an increase in the castrate series as compared to the controls. The relative weight of the thyroid glands of the capons was unaltered from that of the controls even in 80-day-old birds, but histological study of the thyroids of 57-day-old castrated birds revealed a slightly less active gland. The thyrotrophic potency of the pituitary as shown by the assay studies was unaltered.

In view of the very noticeable cytological changes induced in both acidophiles and basophiles by castration, one would expect a much more striking change in one direction or the other in the thyrotrophic activity than was observed. If one may speculate on the negative results obtained here, it would seem likely that the basophiles were not responsible for thyrotrophic activity since even in their great abundance no increase in thyroid activity or pituitary potency was recorded by the methods used. However, the presence of degenerative acidophiles may account for the slight decrease in activity noted in one of the series tested. The following experiments were designed to clarify this problem.

Theelin and Theelin plus limited diet

Daily injection of 0.2 ml. sesame oil containing 500 I.U. estrone into

normal-diet birds from the 3rd to the 19th day inclusive inhibits the development of the basophiles so that the pituitaries at 20 days of age are characterized by the presence of few basophiles and numerous acidophiles (Payne, 1942). Severinghaus (1939) observed degranulation of the basophilic cells in rat pituitaries after estrone injections and stated that estrone activated the secretory cycle of the basophiles to such an extent as to result in final exhaustion. In either case the end result is the presence of agranular non-functioning basophiles. Payne also reported that the number of acidophiles could be further increased if during the injection period the birds were kept on a limited diet, the effect of the two treatments being additive.

The pituitaries of normal-diet birds treated with estrone showed evidence of increased thyrotrophic activity; at the end of the treatment the thyroids weighed nearly twice as much as those of the untreated birds. The significantly heightened follicular epithelium in the thyroids of the treated birds also was indicative of increased activity (Table 2). The thyrotrophic potency of the pituitary as determined by assay was only slightly increased by the estrone injections.

When the estrone was injected into birds kept on limited diet, however, no such increased thyroid activity was recorded; nor did limited diet alone have a significant effect on relative thyroid weight. The thyroid epithelium was unaffected by the treatment. The pituitary thyrotrophic potency was, however, slightly increased by estrone injections in limited diet birds.

The increased thyroid activity and pituitary potency in estrone injected regular diet birds again points to the acidophiles as the active cell type. It is difficult to explain, however, the absence of a response in the estrone injected limited-diet series, unless it be due to a difference in receptivity of the thyroid gland in these birds. A further study of acidophilic pituitaries was made with birds injected with androgens.

Androgen injections

Payne (1942) injected chicks daily with 0.01 mgs. (0.2 ml.) Oreton-B from the 3rd to the 29th day after hatching, and autopsied on the 31st day. A second group of chicks was injected daily with 0.125 (0.2 ml.) Oreton-B from the 5th to the 9th and from the 11th to the 14th day after hatching and was killed on the 20th day. He reported that in each case there was a slight decrease in number of basophiles and that the acidophiles were more prominent. The pituitaries were smaller than those in the controls.

Such a treatment had no significant effect upon the thyroid-pituitary

axis as revealed by the methods used in the present study. The pituitaries of these birds showed no difference in thyrotrophic activity over those of untreated birds (Table 2) and the thyroids of the treated birds showed no significant response to the treatment either as regards weight or histology. This might be simply due to the lack of sensitivity of the methods used. The data cannot be interpreted as conclusive evidence that no change occurred but at least they do suggest that if the pituitaries were altered physiologically, the change was slight as compared with that induced by other treatments reported above. The effect of androgen treatment on the cytology was, in fact, not as great as was that of estrogen injections.

Thiouracil diet

Several workers (Zwecker, 1935; Severinghaus, 1937; Bryant, 1930; Payne, 1944, among others) have described a distinctive type of cell appearing in the anterior pituitary of the mammal and bird after thyroidectomy. There is disagreement among these investigators, however, as to their origin and physiological significance. Severinghaus (1937) supports the idea that they are modified basophiles and are indicative of a degenerate condition. Zwecker (1935) suggested that they arise from chromophobes and are the active thyrotrophin secretory cells. Payne (1944) suggested that they arise from chromophobes and presented evidence showing that they are not synonymous with the castrate basophiles as some investigators have proposed. He ascribed to them a secretory role and implied that they may be responsible for the elaboration of the thyrotrophic hormone. During the course of the present study Payne found numerous thyroidectomy or "T" cells in the pituitaries of 96-day-old birds treated with 0.1% thiouracil for 80 days.

Thyroids of 25-day-old birds were increased enormously in size after only 15 days of 0.1% thiouracil treatment from the 10th to the 24th day (Table 2). This, along with the presence of a heightened epithelium in the thyroids of treated birds suggested a very marked increase in thyrotrophic activity of the pituitary. Assay of the pituitaries from these birds, however, showed no significant difference in potency. Houssay *et al.* (1932) found no change in thyrotrophic potency of rat pituitaries following thyroidectomy. This was confirmed by Smelser (1934) although Payne described the "T" cells as secretory and reports no indication of degenerative changes. If the "T" cell is the thyrotrophin producing cell one might conclude from these data that the increased rate of elaboration is matched by an increased secretory rate which prevents any appreciable storage in the gland.

DISCUSSION

The above data do not reveal a simple answer to the problem. It is difficult to separate such phases of gland activity as elaboration, storage, and mobilization. This must be considered at both the pituitary and thyroid levels. However certain tentative conclusions may be drawn as a basis for further work.

The data as summarized in table 3 do not suggest a basophile origin for the thyrotrophic hormone since the very markedly basophilic pituitaries of the capons showed no increase in potency over those from control birds and the thyroids from the castrated birds were slightly less active than those of control birds. The response to the more strongly acidophilic pituitaries of estrone treated birds suggests some thyrotrophic activity on the part of these cells although the less notably acidophilic pituitaries of androgen treated birds showed no significant change from the normal. The reaction of limited diet birds requires further study but it seems likely that in these animals the receptivity of the thyroid played some part in producing the negative results. The very marked response of the thyroids of thiouracil-treated birds points to the "T" cells as being possibly involved in thyrotrophic activity. These facts suggest a relation-

TABLE 3. SUMMAGY OF CYTOLORY AND THYROTROPHIC ACTIVITY OF PITUITARY

Treatment	Predominant pituitary cell type	Thyroid activity	Pituitary potency
Castration	basophile	−○	○
Estrone	acidophile	+	+
Estrone plus limited diet	acidophile	○	+
Limited diet	acidophile	−○	−○
Androgen	acidophile	○	○
Thiouracil	"T" cells	++	○

− Activity or potency less than in untreated controls.
○ Activity or potency the same as in untreated controls.
+ Activity or potency greater than in untreated controls.

ship between acidophiles and "T" cells. It may be that with a moderate stimulus to the pituitary there is an increase in number of acidophiles. As the demand for thyrotrophic hormone increases a more "active" acidophile is seen in which storage of the hormone can be detected by assay. In extreme situations such as presented by thyroidectomy or thiouracil treatment, the secretory rate, as well as the rate of elaboration is increased so that, with little storage of the hormone, assay shows no increase in potency. This may be the type of cell described as thyroidectomy cells by various cytologists.

SUMMARY

An attempt was made to correlate thyrotrophic activity of the pituitary of the White Leghorn cockerel with its cytology by a histological study of the thyroids and a thyrotrophic assay of pituitaries of variously treated chicks. Treatments were designed to produce pituitaries of a predominant cell type and included limited diet, castration, estrogen injection, estrogen injection with limited diet, androgen injection, and thiouracil feeding. The data are not conclusive but suggest that the basophiles are not involved in the elaboration of the thyrotrophic hormone. The acidophiles are considered as a likely source and it is suggested that the "thyroidectomy" cells should receive serious consideration as a possible modified acidophile.

Acknowledgements

The author wishes to extend his appreciation to Dr. W. R. Breneman for his constant interest and cooperation during the course of this research, and to Dr. W. E. Ricker and Dr. G. W. Snedecor, for their help in the statistical analyses. It must also be emphasized that data reported here on the cytological preparation and interpretation of the pituitaries mentioned in this study are the result of the work of Dr. Fernandus Payne to whom the author is deeply grateful.

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THE ELECTROLYTE-EXCRETING ACTIVITY OF ADRENOCORTICAL SUBSTANCES

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INTRODUCTION

DORFMAN, Potts and Fish (1947) have used the adrenalectomized rats' excretion of administered radiosodium for the detection of small amounts of desoxycorticosterone and have found the radiosodium excretion useful in characterizing the activity of various steroid compounds (Dorfman, 1949). Since Na 24 has a relatively short half-life (14.8 hours) and Na 22 is not easily obtained, it occurred to us that the measurement of urinary sodium and potassium by flame photometry (Overman and Davis, 1947; Shapiro and Hoagland, 1948) might offer certain advantages.

METHODS

The technique of Dorfman *et al.* (1947) involves the collection of urine from radiosodium injected, adrenalectomized rats for six hours following the injection of 0.25 cc. of an oil solution of desoxycorticosterone acetate. A careful study was made of the variables involved in such a procedure when measurement of total urinary Na (and K) is measured instead of radiosodium. It was found that a urine-collection period of 4 hours following DOCA administration was somewhat more accurate provided that certain precautions are observed. The final method evolved was as follows:

(1) Male rats weighing 150 to 175 grams at operation were used for adrenalectomy.

(2) When not in use for assay these animals were maintained with 0.9% NaCl solution as drinking water, but 24 hours previous to the initiation of urine collection tap water was substituted for the saline solution. At three hours previous to the test access to food and water was denied.

(3) Before being placed in the urine-collecting cages complete bladder emptying was assured by holding the head of each animal in a beaker containing ether-soaked cotton until the hind legs showed relaxation (cf. Addis *et al.*, 1949). This method was found preferable to the delivery of a brief electric shock (a few milliamperes and 100 to 400 volts) which caused complete voiding in most (about 80%) but not all shocked animals.

(4) Immediately following the etherization-induced voiding each animal received an intraperitoneal injection of 5 cc. of a 0.9% NaCl solution and a subcutaneous injection of an olive-oil solution of the test steroid. The intraperitoneal saline injection dose was arrived at as optimal after tests of varying NaCl loads.

(5) The animals were then placed in pairs into urine collecting cages which were glass one-gallon jars (with an outside diameter of $6\frac{1}{4}$ inches) having the bottoms re-

Received for publication February 27, 1951.

moved; a one-half inch wire mesh platform inserted above the neck of the inverted bottle held the paired rats, and a cone of fine mesh copper wire inserted below the platform served to catch the feces. For measurement of urine volumes a 15 cc. centrifuge tube was placed under a glass funnel which circumscribed the opening of the inverted gallon jar.

(6) Completeness of collection of all excreted urine was assured by removing any feces present, and washing the collection bottle and funnel with 210 cc. of distilled water. These washings combined with the collected urine did not over-dilute the contained electrolytes.

Urinary sodium and potassium determinations were made with the Perkin-Elmer flame photometer (model 52A).

TABLE 1. URINE VOLUMES, NA AND K EXCRETION AT VARIOUS DOCA DOSAGES*

Dosage in μg.	No. of pairs of rats	Urine Volumes				Sodium				Potassium			
		cc./ 4 hrs.	S.D.	S.E.	% De-crease	Mg./ 4 hrs.	S.D.	S.E.	% De-crease	Mg./ 4 hrs.	S.D.	S.E.	% In-crease
Oil injected	19	3.55	.977	.219	—	6.12	.291	.067	—	4.48	.210	.047	—
2.4	7	3.41	.460	.174	0.1	5.88	.454	.171	9.2	5.00	.249	.092	11.9
6.0	12	2.77	1.120	.321	20.5	4.24	.262	.076	29.7	5.52	.196	.057	25.6
12.0	6	2.28	.688	.281	33.1	2.88	.171	.070	55.4	6.08	.386	.158	30.5
30.0	11	2.16	.777	.234	41.1	2.44	.182	.055	59.8	6.40	.362	.109	43.2
60.0	11	1.74	.616	.186	49.9	1.40	.115	.035	77.5	6.20	.462	.128	38.2
120.0	5	1.44	.224	.100	56.7	1.60	.185	.083	75.7	6.68	.220	.098	51.9

$$\text{S.D.} = \pm\sqrt{\frac{\Sigma d^2 f}{n-1}}. \qquad \text{S.E.} = \pm\sqrt{\frac{\Sigma d^2 f}{n(n-1)}}.$$

RESULTS

Desoxycorticosterone acetate assay

In Table 1 we present the urine volume, sodium and potassium excretions observed in control and DOCA-injected animals. Using a logarithmic scale the dosage-response curves for the electrolytes are plotted in Figure 1 and that for urine volume changes in Figure 2. With the numbers of rat pairs employed significant output changes over the controls occur at the 6 μg. dose and above in both Na and K excretions and at the 12 μg. dose and above in the antidiuretic effect. The steepness of the Na dosage-response curve and its relatively low variability (compare the standard deviations in Table 1) indicates that this single measurement is the most accurate for assay purposes. We have analyzed possible combinations of these data for the construction of a multivariate assay curve, but no notable increase in accuracy is obtained.

The data of Table 1 were assembled from five separate experiments in which the number of rat pairs employed varied from 7 to 13. Analysis of the slope constant (b) of each of these taken individually discloses values of b ranging from .039 to 0.774 for the K excretion and -0.396 to -1.047 for Na excretion. The index of accuracy (λ, or s/b) varies from .439 to 7.564 for K excretion and from $-.108$ to $-.583$ for Na excretion. The greater accuracy of the Na excretion data are emphasized. When the

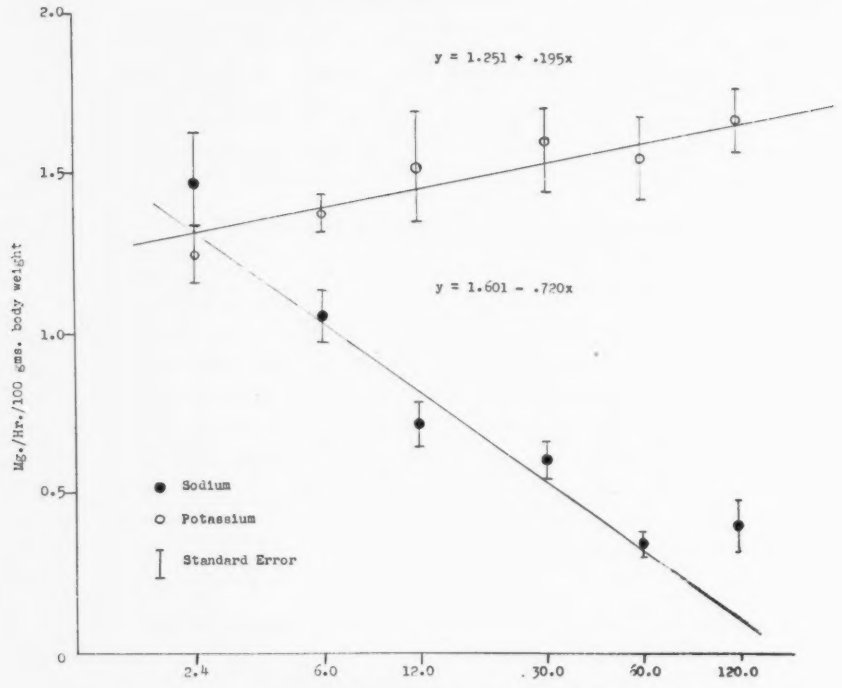

FIG. 1. Dosage Response Curve for 4-Hour Assay.

data for all the 52 rat pairs are similarly analyzed b and λ for K excretion are 0.200 and 1.575 and for Na −.703 and −.361. With an adequate number of rat pairs, an accuracy comparable to that of most standard bioassays is obviously obtainable on the basis of Na retention.

The decline in urine output is positively correlated with the Na output decline as indicated by the data of Figure 3. It is nonetheless notable that over the dosage range employed the percentage of Na excretion-reduction is in every instance greater than the percentage change in urine volume, and the rate of change in Na output with increasing dosage is greater (i.e. the slope of the regression line of Figure 3 is less than 1 and the intercept is not at 0).

Assay of adrenal extract and pure steroids

In Table 2 we present the assay data collected for 11-dehydrocorticosterone, corticosterone, 11-dehydro17-hydroxycorticosterone, 17-hydroxycorticosterone, Δ^5-pregnenolone and lipo-adrenal extract. These are compared to the mean data for two dosages (6 and 60 μg.) of DOCA

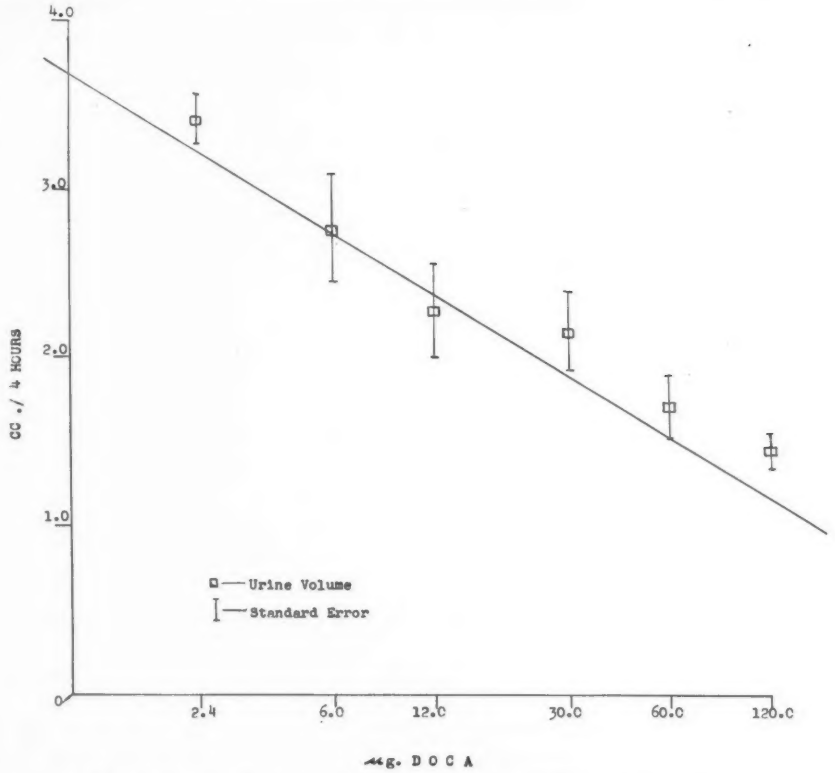

FIG. 2. Dosage Response Curve of Urine Excretion in 4-Hour Assay.

which were employed simultaneously with the various sets of tests of the other substances. The dosage:response data for Na and K are plotted in Figures 4 and 5.

All the substances (except possibly corticosterone) caused Na retention and reduction in urine volume at the 10 microgram dose, but at the higher doses employed differences between the effects of various compounds are obvious. Thus lipo-adrenal extract alone resembles DOCA in inducing Na retention over the entire dosage-range employed; it is antidiuretic over a considerable range, but exerts a diuretic effect at the highest dose employed (500 µg. cortisone equivalent). Nonetheless, the slopes of the Na-dosage-response curves for lipo-adrenal extract and DOCA differ significantly ($<5\%$ level of confidence). With the exception of pregnenolone, the crystalline steroids tested exhibit sodium and water-

TABLE 2. EFFECT OF ADRENOCORTICAL SUBSTANCES ON Na, K AND URINE EXCRETION

Substance injected	Dosage in μg.	No. of pairs of rats	Na mg./hr.	S.E.	K mg./hr.	S.E.	Urine volume cc./4 hrs.	S.E.
DOCA	oil	19	1.90	.085	1.28	.059	4.09	.207
	6	18	1.09	.070	1.57	.047	3.23	.203
	60	15	0.39	.041	2.06	.139	2.14	.230
Dehydro-corticosterone	10	4	0.93	.400	1.19	.140	2.08	.698
	50	4	2.04	.274	1.74	.142	3.30	.601
	200	3	1.90	.265	1.82	.017	4.27	.035
Corticosterone	50	4	1.79	.189	1.69	.152	4.58	.623
	200	4	2.34	.143	2.52	.089	8.20	.408
	400	3	2.36	.337	3.16	.215	10.85	1.110
17-Hydroxy-dehydrocor-ticosterone	10	4	1.69	.287	1.56	.089	3.85	.741
	50	4	2.00	.053	1.46	.050	4.90	.271
	200	3	2.69	.154	2.20	.121	9.33	.501
17-Hydroxy-corticosterone	10	4	1.48	.318	1.24	.107	3.36	.677
	50	4	2.26	.213	1.59	.170	4.48	.609
	200	3	2.09	.004	1.97	.120	4.43	1.028
Lipo-Adrenal Cortex	10	4	1.23	.146	1.47	.104	3.00	.497
	100	4	0.77	.123	1.61	.100	2.68	.403
	500	3	0.64	.098	3.30	.128	6.50	.896
Pregnenolone	10	4	1.58	.124	1.36	.072	3.40	.716
	100	4	1.53	.089	1.20	.070	2.88	.629
	500	4	1.65	.134	1.28	.132	3.85	.896

diuretic effects at the higher dosages employed, corticosterone and cortisone being the most effective in this respect.

Increased potassium excretion is effected by all substances tested except Δ^5-pregnenolone, but some of the dosage-response curves obtained differ in slope from that of DOCA (lipo-adrenal extract, corticosterone). And the potency in K-excretion effect of those substances having a similar dosage-response curve to DOCA is about one-tenth that of DOCA.

DISCUSSION

The assay method as outlined appears to have a sensitivity suitable for the detection of DOCA over a range of 6 to 60 μg. using Na as the indicator, and over a range of 6 to 30 μg. using K as the indicator. Even the urine volumes seem to demonstrate a rather close relationship to DOCA dosages. As the statistical analysis suggests, Na retention, under these conditions, gives the most reliable and accurate assay. It should be noted, however, that the assay involves a standard Na load. It would be of interest to determine if a standard K load would increase the accuracy of the K dosage-response data.

While only lipo-adrenal cortex showed any similarity to DOCA by consistently causing Na retention, the K and urine volume data enhance

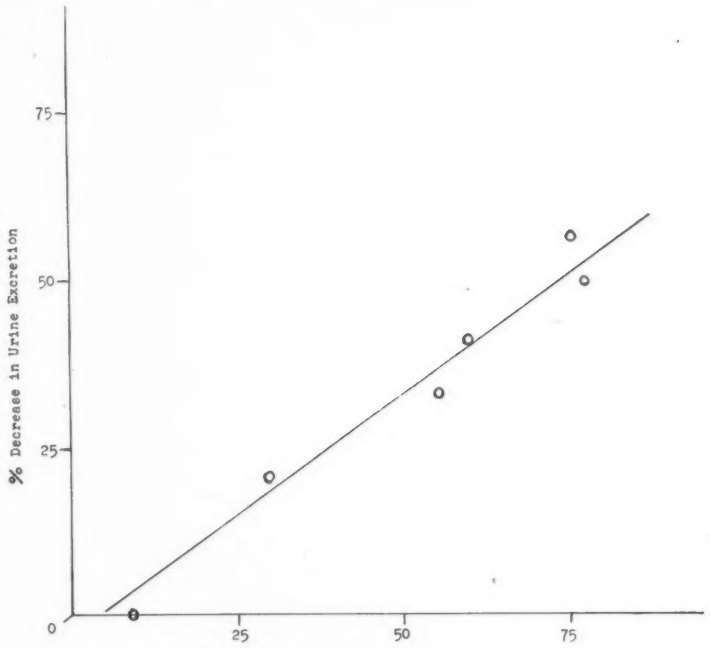

FIG. 3. % Decrease in Na Excretion.

the differentiation between the two. Excluding pregnenolone, the four corticosteroids tested had excretion effects similar to one another, but significantly different from those of DOCA at the 50 μg. and greater dose levels.

SUMMARY

Using the flame photometer for determination of electrolytes excreted in adrenalectomized rat urines, a method has been devised for the bioassay of adrenal cortical substances which is sensitive over a range from 6 to 60 μg. of DOCA equivalents. Cortisone, 17-OH corticosterone, corticosterone, and 11-dehydrocorticosterone differ qualitatively from DOCA in this test. Pregnenolone has no significant effect on the variables studied, and lipo-adrenal extract has a DOCA-like effect, but is not as marked an antidiuretic and sodium retainer.

Acknowledgments

The desoxycorticosterone acetate used in these experiments was supplied by the Ciba Co., Ltd. We are indebted to Dr. E. C. Kendall of the Mayo

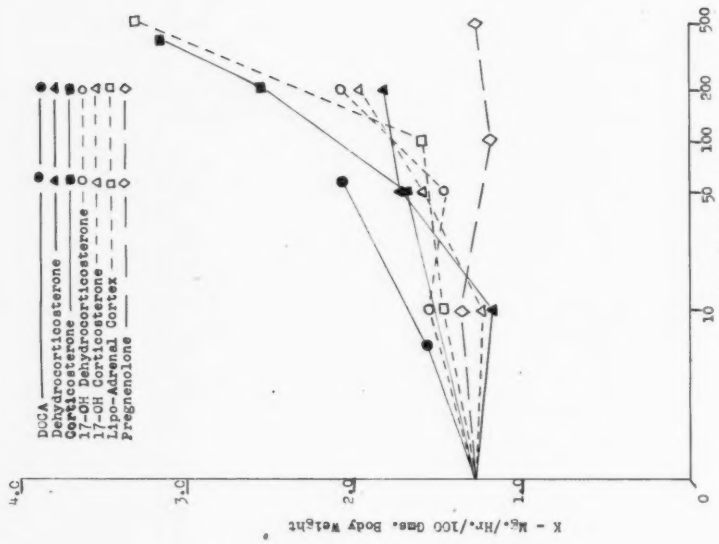

FIG. 5. Effect on K Excretion of Some Adrenocortical Substnaces.

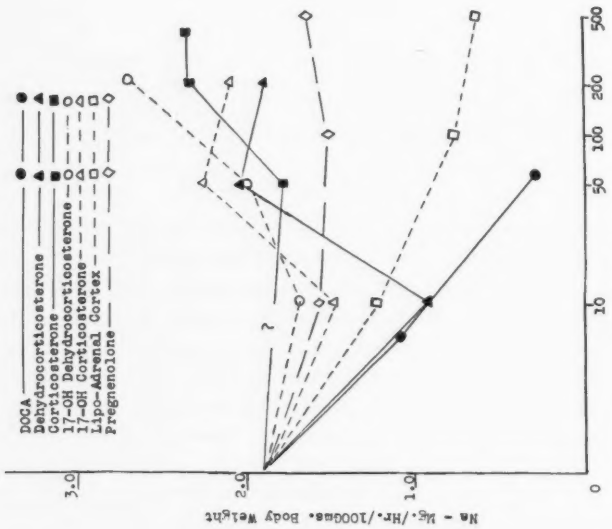

FIG. 4. Effect on Na Excretion of Some Adrenocortical Substances.

Clinic for the 11-oxygenated steroids and to the Upjohn Co. for the lipoadrenal extract. Grant aid was supplied by the Ciba Co. and the U. S. Public Health Service (RG-999).

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GONADOTROPHIC ACTIVITY OF GRANULE FRACTIONS OBTAINED FROM ANTERIOR PITUITARY GLANDS OF CASTRATE RATS[1]

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DATA collected during recent years show that succinoxidase, cytochrome oxidase and adenosine triphosphatase are associated with the large granule fraction isolated by differential centrifugation of rat liver homogenates. Only a few enzymes are associated with the small granules, but these particles contain a large part of the pentosenucleic acid of the tissue. The granules remain morphologically intact in sucrose solutions of the proper concentration and this makes these solutions preferable to water, isotonic sodium or potassium chloride solutions for isolation purposes (Schneider, 1946; Hogeboom, Claude and Hotchkiss, 1946; Hogeboom, Schneider and Pallade, 1948; and Schneider, Claude and Hogeboom, 1948). The work in this field is summarized in a review by Schneider and Hogeboom (1951).

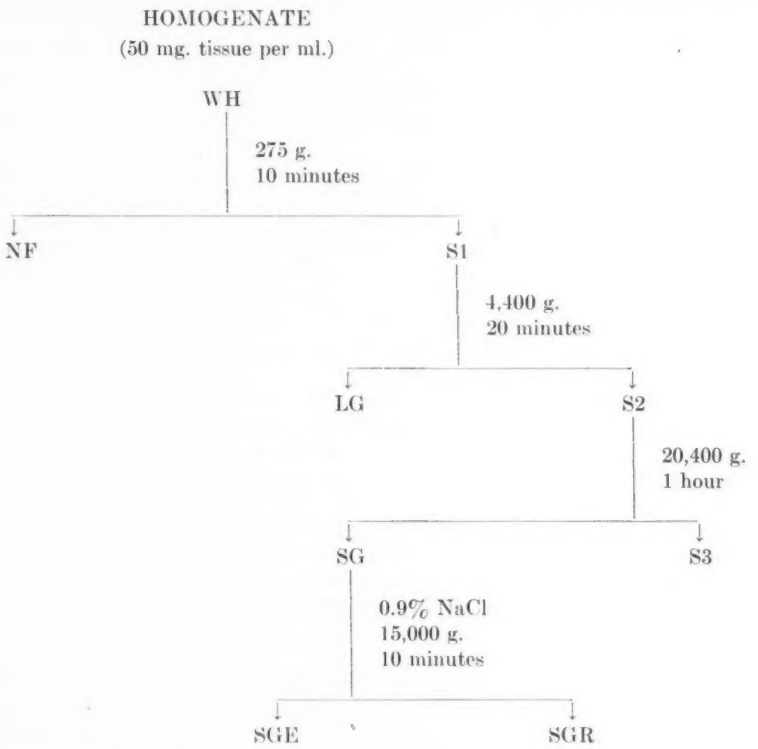
Preliminary results have also been reported on the fractionation of sucrose homogenates of rat pituitary glands by differential centrifugation, the gonadotrophic hormone content and the succinoxidase activity of these fractions (McShan and Meyer, 1949). Over 50 per cent of the gonadotrophin was associated with the large and the small granule fractions and approximately 60 per cent of the succinoxidase activity was associated with the large granules. Further work on this problem has resulted in the development of a method by which the major part of the gonadotrophin of rat pituitary glands is recovered in one granule fraction which consists of small granules. This fractionation procedure and the results of the biological study of the various fractions obtained are given in this report.

MATERIALS AND METHODS

Adult female rats of the Holtzman strain were castrated and used as donors of anterior pituitary glands for the fractionation experiments. Adult female rats were used because they were available in large numbers and it is known that the gonadotrophin

Received for publication August 10, 1951.

[1] Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and by a research grant from the National Institutes of Health, Public Health Service.



Designations of fractions:

WH Whole homogenate
NF Nuclear fraction
LG Large granule
SG small granule
SGE Small granule extract
SGR Small granule residue
S1 Supernatant fraction, 275 g.
S2 Supernatant fraction, 4,400 g.
S3 Supernatant fraction, 20,400 g.

DIAGRAM 1. Procedure for fractionation of sucrose homogenates of anterior pituitary glands from castrate rats.

increases to high levels in the anterior pituitary glands of these animals within a short time after castration (Hellbaum and Greep, 1940). The minimum time of castration before using the animals as donors was three weeks, and the maximum time was six months. As was to be expected the glands from these animals contained a high level of gonadotrophic activity. Furthermore, the glands from rats can be removed almost immediately after the animals are killed, and homogenized within a short time. This is advantageous in that there is little time for tissue changes to occur.

A few homogenates were made with glands from young adult female and adult male

rats, but these glands were unsatisfactory as they contained little gonadotrophin. These animals were not available in sufficient numbers to justify their use after castration. Glands from normal young adult male rats contained considerable gonadotrophin but these animals were also not available in sufficient numbers. Pituitary glands of cows, hogs, horses and sheep were homogenized in sucrose and fractionated but satisfactory separations were not obtained.

Groups of 30 to 165 rats were used for each run. The pituitary glands were removed in the cold room immediately after killing the animals, and were kept in a cold moist chamber until all glands were removed. The anterior lobes were separated, weighed and placed in a sharp-pointed homogenizing tube that was kept in an ice bath except during homogenization. The anterior lobes were homogenized immediately in 19 volumes of 0.88 M sucrose solution. A few homogenates were made using 0.154 M KCL. Similarly 19 volumes of KCl were used in making these homogenates. The average weight of the anterior lobes obtained from over 1600 rats that had been castrated for 3 weeks to 6 months was 11.14 mg.

The pituitary homogenates were separated into nuclear, large granule, small granule, and supernatant fractions by differential centrifugation in the multispeed head of a refrigerated International centrifuge which was kept at approximately 2° C during centrifugation. The final fractionation procedure given in Diagram 1 is the result of information obtained from several different experiments. The method is given in detail as follows:

1. The whole homogenate (WH) was centrifuged at 275 g. for 10 minutes. The opaque supernatant extract (S1) was removed with a 1 ml. syringe fitted with a 22 gauge needle, or with a medicine dropper drawn out to a fine point. In some experiments the residue was washed by suspending in 0.88 M sucrose and centrifuging for 10 minutes at the same speed. The washings from the residue were combined with S1. The residue (NF) consisted of nuclei and other insoluble material.

2. The supernatant (S1) was centrifuged at 4,400 g. for 20 minutes. The resulting sucrose supernatant (S2) was carefully removed. The large granule fraction (LG) was suspended in 0.88 M sucrose and recentrifuged. It was found, however, that washing the LG fraction removed little gonadotrophin, and in the later work it was abandoned.

3. S2 was centrifuged at 20,400 g. for 1 hour to give the final supernatant fraction (S3) and the small granule fraction (SG). The SG fraction was extracted by mixing thoroughly with 0.9 per cent NaCl solution and centrifuging at 15,000 g. for 10 minutes. This extraction process was repeated two to there times and the extracts were combined to give the SGE fraction. In the recent work the SG fraction was extracted eight times. The extracted granules were designated as SGR.

The fractions obtained by the above procedure were stained for mitochondria by use of fast green FCF as reported by Harman (1950).

The nitrogen determinations were done by a modification of the micromethod of Johnson (1941).

The homogenates and fractions were assayed for gonadotrophic hormone activity by use of 21 day old female rats of the Holtzman strain. The volumes of the fractions were adjusted with normal saline so that each rat received 0.5 ml. of the proper fraction on the afternoon of the first day, and 0.5 ml. on the morning and afternoon of the next four days. Autopsy was performed on the the morning after the last injection. The overies were removed, dissected free of other tissues, and weighed. The presence of follicles and corpora lutea was recorded. The degree of stimulation of the uteri was also noted. They were enlarged in most cases but usually were not distended with fluid.

TABLE 1. GONADOTROPHIC ACTIVITY OF FRACTIONS ISOLATED FROM ANTERIOR PITUITARY GLANDS OF CASTRATE FEMALE RATS

Fraction	Speed of centrif.	No. of runs	No. of rats	Total dose*	Weight ovaries
	g.			mg.	mg.
Whole homogenate		2	6	10	235
Whole homogenate		2	6	20	216
Nuclear fraction	400	1	3	10	26
Nuclear fraction	400	2	6	20	28
Large granules	8,000	2	6	10	61
Large granules	8,000	2	6	20	75
Small granules	20,400	2	6	10	46
Small granules	20,400	2	10	20	195
Supernatant fraction	20,400	2	5	10	51
Supernatant fraction	20,400	2	7	20	121

* The doses given in this and the following tables are in terms of milligrams equivalent of fresh anterior pituitary tissue.

RESULTS AND DISCUSSION

The results given in Table 1 were obtained by fractionating 0.88 M sucrose homogenates of anterior pituitary glands of castrate female rats. The gonadotrophic activity as reported previously (McShan and Meyer, 1949), was associated with the two granule fractions, one obtained at 8,000 g. and the other at 20,400 g. Since under these conditions of centrifugation the gonadotrophic activity associated with the granules was not recovered in a single fraction, further experiments were done using sucrose and potassium chloride homogenates to determine the conditions for recovering the major part of the gonadotrophin in a single granule fraction. Homogenates were fractionated, therefore, by centrifuging at increasing speeds as shown in Table 2. The two kinds of homogenates and the

TABLE 2. FRACTIONATION OF SUCROSE AND POTASSIUM CHLORIDE HOMOGENATES OF ANTERIOR PITUITARY GLANDS FROM CASTRATE FEMALE RATS

Fraction	Speed of centrif.	No. of runs	Total dose	Sucrose (0.88 M)		Potassium chloride (0.154 M)	
				No. of rats	Weight ovaries	No. of rats	Weight ovaries
	g.		mg.		mg.		mg.
Whole homogenate		2	40	6	245	6	230
Nuclear fraction	275	2	40	5	26	6	19
Granules	2,400	2	40	6	29	6	183
Granules	4,400	2	40	6	37	6	147
Granules	6,900	2	40	6	35	6	32
Granules	9,900	2	40	6	43	6	21
Granules	13,500	2	40	6	47	4	19
Granules	20,400	2	40	6	129	4	17
Supernatant fraction	20,400	2	40	6	163	6	119

fractions obtained from them were assayed at the 40 mg. level in order to obtain information on all the fractions even though some of them might be low in gonadotrophic activity.

The results given in Table 2 show that little gonadotrophin was associated with the granule fractions separating from the sucrose homogenates at speeds below 6,900 g. whereas the major part of the activity was recovered at speeds ranging from 6,900 g. to 20,400 g. In contrast to these results, the major part of the gonadotrophin associated with the granules of 0.154 M KCl homogenates was recovered at 2,400 g. and 4,400 g. whereas little activity was obtained with the granules separating at speeds ranging from 6,900 g. to 20,400 g. It is clear that the gonadotrophin of sucrose homogenates was associated with granules recovered at high speeds, but in the case of KCl homogenates most of the hormone associated with the granules was recovered at relatively low speeds of centrifugation. This latter result is presumed to be due to a difference in specific gravity of the suspending fluids, and to aggregation or clumping of the granules in the presence of KCl. The granules from liver homogenates made with NaCl and KCl are known to aggregate (Hogeboom, Schneider and Pallade, 1948; Schneider and Hogeboom, 1951). The nuclear fractions from the two kinds of homogenates contained little activity. Considerable gonadotrophic activity, however, was found in the supernatant fractions obtained by centrifuging the sucrose and KCl homogenates at 20,400 g. as indicated by average ovarian weights of 163 mg. and 119 mg., respectively (Table 2).

On the basis of the results of Table 2 it was decided to isolate two granule fractions from sucrose and KCl homogenates by centrifuging at 4,400 g. and 20,400 g. It was presumed that the major part of the gonadotrophin associated with the granules of KCl homogenates would be obtained at 4,400 g. whereas the hormone associated with the granules of sucrose homogenates would be recovered by centrifuging the fraction obtained

TABLE 3. FRACTIONS FROM SUCROSE AND POTASSIUM CHLORIDE HOMOGENATES OF ANTERIOR PITUITARY GLANDS FROM CASTRATE FEMALE RATS

Fraction	Speed of centrif.	0.88 M sucrose*			0.154 M KCl*		
		No. of rats	Total dose	Weight ovaries	No. of rats	Total dose	Weight ovaries
	g.		mg.	mg.		mg.	mg.
WH (Whole homogenate)		9	20	207	3	20	184
Granules†	4,400	9	20	31	2	20	161
SG (Small granules)	20,400	9	20	161	3	20	17
S3 (Supernatant fraction)	20,400	6	20	133	3	20	141

* There were three runs using sucrose homogenates and one using a KCl homogenate.
† The fraction obtained from the KCl homogenate at 4,400 g. contained both large and small granules.

TABLE 4. FRACTIONS OBTAINED FROM SUCROSE HOMOGENATES OF ANTERIOR PITUITARY GLANDS FROM CASTRATE FEMALE RATS

Fraction	Speed of centrif.	No. runs	No. rats	Total dose	Weight ovaries	Protein/ mg. FT.*	Protein % of total	Wt. ovaries/ mg. protein inj.
	g.			mg.	mg.	mg.		
WH		1	3	5	61	0.201 (3)†	100	
		5	15	10	176			
		3	9	20	207			51.4
NF	275	2	6	20	28	0.053 (2)	26.4	26.4
LG	4,400	4	12	10	24	0.031 (4)	15.2	
		3	9	20	31			50.0
SG	20,400	5	15	10	80			
		3	9	20	161			241.0
SGR		5	15	10	20	0.022 (4)	11.0	
		2	6	20	33			75.0
SGE		5	15	10	52	0.011 (4)	5.4	
		2	6	20	107			500.0
SGR+SGE		5	15	10	73			
		1	3	20	176			
S3	20,400	4	12	10	37	0.095 (3)	47.1	
		2	6	20	133			70.2
Granules	150,000	1	3	20	19	0.017 (1)		
		1	3	40	33			
Supernatant	150,000	1	3	10	42	0.078 (1)		

* FT indicates fresh tissue.
† Indicates number of preparations used for determinations. Recovery of protein was 105%.

at 4,400 g. at the higher speed of 20,400 g. The results given in Table 3 substantiate this assumption. Since most of the gonadotrophin associated with the granules of sucrose homogenates was recovered in the small granule fraction obtained at 20,400 g., homogenates made with 0.88 M sucrose were used in the remainder of the work reported in this paper.

On the basis of the above results several homogenates were fractionated by centrifuging at 275 g., 4,400 g. and 20,400 g. as outlined in the procedure of Diagram 1. The homogenates and the fractions obtained from them were assayed by administration of two doses, namely, 10 mg. and 20 mg., and the data are summarized in Table 4. Little activity was found in the NF and LG fractions, but a large percentage of the gonadotrophic activity was associated with the SG fraction and considerable activity, as was indicated by the results of previous experiments, was found in the S3 fraction. The SG fraction was extracted three to four times with 0.9 per cent NaCl solution which removed much of the gonadotrophin associated with these particles. When these washings (SGE) were assayed at the 20 mg.

level, they produced ovaries that had an average weight of 107 mg. as compared to 161 mg. for the SG fraction and 33 mg. for the SGR fraction.

In order to determine whether the activity of the S3 fraction was associated with still smaller particles, one supernatant fraction obtained at 20,400 g. was centrifuged at 150,000 g. for 1.5 hours. A small amount of solid material was recovered but, as shown by the results (Table 4), it contained little gonadotrophic activity. Furthermore, the activity of the supernatant was not changed significantly by removal of this solid material. These results show that the major part of the gonadotrophic activity of the supernatant fraction is not associated with particles that are removed at 150,000 g., and this makes it appear that the gonadotrophin of this fraction is in solution.

The homogenates (Table 4) contained nitrogen equivalent to 0.201 mg. of protein per mg. of fresh tissue. The protein content of each of the fractions is given in column 7 of Table 4 and the percentage values are given in column 8. It is seen that the SGE which has high gonadotrophic activity contained 0.011 mg. protein per mg. of fresh tissue, or 5.4 per cent of the total protein. The values given in the last column of Table 4 were obtained by dividing the ovarian weights by the protein content of the 20 mg. dose level. Although these values are not directly proportional to each other, they do give some indication of the comparative activity of the various fractions in relation to their protein content. On this basis the SGE is more than ten times as active as the fresh tissue. The results of Table 4 support the conclusion that a high percentage of the gonadotrophin of rat pituitary glands is associated with particles of the small granule fraction, and that a major part of this activity is removed by extracting these particles with isotonic saline solution. When the sodium chloride is removed by dialysis a precipitate forms with a concomitant loss in activity.

The homogenates used in these studies were prepared by grinding or homogenizing in a sharp-pointed ground glass homogenizer. It was thought that at least a part of the gonadotrophic activity of the S3 fraction might have been "solubilized" during the homogenizing process. For purposes of obtaining information concerned with this possibility six homogenates were made by mashing the tissue in the homogenizer with a minimum of grinding. The results of the assays of these homogenates and their supernatant fractions obtained at 20,400 g. are given in Table 5. The data obtained on assaying the S3 fractions from several homogenates that were made by grinding are used for comparison. These results show that the S3 fractions contained less gonadotrophic activity when the homogenates were made by the mashing procedure, and suggest that

gonadotrophin was "solubilized" by grinding. This is shown by the fact that at the 20 mg. level the average ovarian weight of 59 mg. was obtained for the supernatant fractions resulting from the mashing procedure as compared to an average weight of 124 mg. for the corresponding fractions obtained from homogenates made by grinding.

On the basis of the data given in Table 5 for the homogenates made by mashing, it is estimated that 70 to 75 per cent of the total gonadotrophic

TABLE 5. GONADOTROPHIC ACTIVITY OF FRACTIONS OBTAINED BY HOMOGENIZING AND BY MASHING

Fraction	Speed of centri.	Total dose	Homogenate made by:	
			Grinding	Mashing
			Weight of ovaries	
	g.	mg.	mg.	mg.
WH	—	20	207 (9)*	194 (18)*
NF	275	20	28 (6)	35 (6)
LG	4,400	20	31 (9)	36 (15)
SG	20,400	20	161 (9)	145 (12)
S3	20,400	10	41 (17)	30 (3)
S3		20	124 (13)	59 (18)

* Figures in parentheses indicate number of rats used for the assays of the fractions.

activity of the glands of castrate rats was associated with the SG fraction. Thus, an average ovarian weight of 145 mg. was produced by the small granule (SG) fraction as compared to 194 mg. for the whole homogenate (WH). The other fractions contained relatively little activity as indicated by small ovarian weights of 59, 35, and 36 mg., respectively, for the S3, NF and LG fractions. The activity present in the NF and LG fractions is probably contamination due to a lack of complete separation during centrifugation and, in the case of the residue, to small particles of intact cells that were not completely homogenized. Furthermore, as stated above, the results of Table 5 show that part of the hormone found in the S3 fraction was "solubilized" during homogenization. It appears, therefore, on the basis of these results that even more than 75 per cent of the gonadotrophin of castrate rat pituitary glands was associated with the SG fraction.

As indicated by the staining method of Harman (1950) some mitochondria were found in the SG fraction. It is of interest that the mitochondria of this fraction appeared to be smaller than those of the LG fraction. The concentration of the mitochondria in the various fractions was not quantitatively estimated, but it is presumed that they were concentrated in the LG fraction since this fraction obtained from other experiments was high in succinoxidase activity. It should be pointed out in this connection that 60 per cent of the succinoxidase of rat pituitary

glands was shown previously to be associated with the LG fraction (McShan and Meyer, 1949), and that the succinoxidase of rat liver is also associated with mitochondria (Schneider and Hogeboom, 1951). Thus, the mitochondrial or large granule fraction has been shown to contain a high level of succinoxidase and, as indicated above, it was low in gonadotrophic activity whereas the SG fraction contained as much as 75 per cent of the total gonadotrophin and data from other experiments show that it contained little succinoxidase. In the light of these results it appears that the high gonadotrophic activity of the SG fraction is not due to the mitochondria.

The question arises as to whether the gonadotrophin of castrate rat anterior pituitary glands is produced by the small particles with which it is associated, or whether it is produced elsewhere in the cell and is later adsorbed on these particles. The present data do not indicate which of these possibilities represents best the situation in the cell.

Results obtained from experiments on augmentation show that the SGR and SGE augment the action of FSH made from sheep pituitary glands by tryptic digestion (McShan and Meyer, 1940), but these fractions do not augment chorionic gonadotrophin. The results obtained with FSH indicate that the SGE and SGR fractions contain luteinizing hormone activity. The failure to obtain augmentation with chorionic gonadotrophin may be due to the possibility that these pituitary fractions contain sufficient luteinizing hormone to augment them completely. The various pituitary fractions when given alone produced ovaries that contained both follicles and corpora lutea.

SUMMARY

A method is presented for the fractionation of sucrose homogenates of anterior pituitary glands from castrate female rats into nuclear residue, large granule, small granule and supernatant fractions. As much as 70 to 75 per cent of the gonadotrophic hormone activity was recovered in the small granule fraction. The amount found in the supernatant fraction depends on whether the homogenate is made by grinding or by mashing. The nuclear residue and the large granule fractions contain little activity.

The major part of the gonadotrophin associated with the small granules is separated by extracting them with isotonic saline solution. This fraction contains both follicle stimulating and luteinizing activities. The nature of the particles with which the hormone is associated is not revealed by the present data. It is postulated, however, on the basis of present results that the gonadotrophin is not associated with the mitochondria.

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THE IDENTIFICATION OF PITUITARY INTERSTITIAL CELL STIMULATING HORMONE IN HUMAN URINE

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THE purpose of this communication is to describe a method for the preparation and identification of pituitary interstitial cell stimulating (or luteinizing) hormone in human urine.

Presumptive evidence for the presence of interstitial cell stimulating hormone (ICSH) in urine extracts has existed for many years. The presence of ICSH is implied in all extracts which give a positive test for gonadotropins by conventional bio-assay procedures depending upon uterine or ovarian enlargement, since ICSH is necessary for the completion of follicular growth and for estrogen secretion. However, a female test animal cannot at present be employed for a quantitative assay of either follicle stimulating hormone (FSH) or ICSH (1) in an unfractionated gonadotropin preparation, such as a urine concentrate, because in this sex the two hormones appear to be inextricably related in the expression of their physiological effects. It was not until the demonstration that in the male animal synergism between FSH and ICSH does not pertain to all structures responding to these hormones that the development of an actual assay for ICSH became theoretically possible (Shedlovsky, Rothen, Greep, van Dyke and Chow, 1940; Greep, van Dyke and Chow, 1941 and 1942). These workers showed that the augmented androgen secretion resulting from ICSH stimulation of testicular interstitial tissue produces an increase in the weight of the accessory sexual organs of the hypophysectomized male rat. The ventral lobe of the prostate gland was found to be a sensitive and, more importantly, a specific indicator of ICSH effect, its weight not being influenced by the simultaneous administration of biologically pure FSH. In a series of independent studies, investigators at the University of California obtained similar results (Fraenkel-Conrat, Li, Simpson and Evans, 1941; Simpson, Li and Evans, 1942a, b).

Received for publication October 5, 1951.

This work was supported by a grant from the Commonwealth Fund of New York and was aided by a grant from the Playtex Park Research Institute to the Children's Medical Service at the Massachusetts General Hospital.

The necessity of employing hypophysectomized rats for unequivocal demonstration of the presence of ICSH and the comparatively minute quantity of this hormone excreted by the human being have combined to impede the application of these observations to the detection of ICSH in human urine. So severely has the toxicity of urinary concentrates limited the quantity of material which could be injected into the test animal that only fragmentary and conflicting results have hitherto been obtained.

In an unpublished experiment, Tyndale (quoted by Engle, 1939) succeeded in producing hypertrophy of the testicular interstitial tissue and an increase in the weight of the genital tracts of hypophysectomized rats injected with large doses of concentrates prepared from the urine of castrate women; Greep and Jones (1950) obtained similar results with the urine of post-menopausal women. Evans and Gorbman (1942) found that repair of the ovarian interstitial tissue of hypophysectomized female rats could be effected by the administration of concentrates of the urine of males. Varney, Kenyon and Koch (1942) administered concentrates of the urine of a patient with ovarian agenesis to hypophysectomized male and female rats and observed that the highest dosage produced a slight stimulation of the interstitial cells and enlargement of the seminal vesicles in the males and the development of a few corpora lutea in the females. Smith, Engle and Tyndale (1933), on the other hand, failed to detect any ICSH in urine from castrate and menopausal women. Jones and Bucher (1943) also found the urine of menopausal women, normal men and of normally menstruating women to be devoid of ICSH activity.

In our hands, urine extracts prepared by the conventional alcohol precipitation or ultrafiltration techniques proved toxic to hypophysectomized animals in doses too small to bring about consistent physiological effects. The work reported here is based upon the use of a chromatographic method for the preparation of non-toxic concentrates of urine.

METHODS AND MATERIALS

Urine obtained from young women was pooled, refrigerated and concentrated as soon as possible after collection according to the technique of Bradbury, Brown and Brown (1949). Although variations in the rate of ICSH excretion presumably occur during the different phases of the menstrual cycle, pools comprising specimens of urine collected during all the intermenstrual phases proved to contain sufficient amounts of ICSH for methodological studies. The ICSH potency of successive pools was found to be more closely correlated with the number of hours' output of urine than with the urine volume. A careful record was therefore kept of the number of hours' output represented by each specimen of urine added to the pool, enabling the dose of urinary concentrate administered to a test animal to be expressed in terms of the number of hours' output to which it is equivalent.

The urine was acidified to pH 4.5 with glacial acetic acid and run through a coarse filter to remove particulate matter. An aqueous suspension of kaolin (5 cc. of a 20% suspension to each 100 cc. of the acidified urine) was then added and after thorough stirring was allowed to settle out. The supernatant fluid having been decanted and discarded, the kaolin was resuspended in water by means of vigorous shaking. After centrifugation, the supernatant fluid was again decanted and discarded. In this manner, the kaolin was washed repeatedly with water until it was entirely free of urine. The gonadotropins were twice eluted from the kaolin by adding volumes of 1 N ammonium hydroxide equal to that of the kaolin suspension originally added to the urine. The pH of the combined eluates was initially lowered to 8.5 by the addition of glacial acetic acid and if any precipitate appeared at this point it was discarded. The pH was then reduced to 5.5 by further additions of glacial acetic acid, and the gonadotropins precipitated from the solution by the addition of four volumes of 95% ethanol. The precipitate was dried in a vacuum desiccator and dissolved in a volume of water convenient for injection.

Weanling male rats of the Sprague-Dawley strain which had been hypophysectomized at a body weight of 45 to 55 grams were employed as the test animsls. They were injected subcutaneously with 0.5 cc. of a solution of the concentrate twice daily for four days, beginning on the fifth day after hypophysectomy. (Regardless of the dose of concentrate being administered, the dilution was such that the total volume of fluid injected into each rat was 4 cc.) The animals were killed on the day following the last injections and the testes, seminal vesicles and ventral prostate removed. After being freed of fat and fibrous tissue these organs were weighed immediately on a Roller-Smith torsion balance.

The testes were prepared for histological examination by fixation in Zenker acetic acid. Sections cut at a thickness of seven μ were stained with: (a) hematoxylin and eosin, (b) Masson's trichrome, (c) Mallory's phosphotungstic acid hematoxylin, (d) phloxine-methylene blue, and (e) Verhoeff's elastic tissue stain. For the histochemical studies the testes were fixed in aqueous ten per cent formalin for one week, washed thoroughly in running tap water and sectioned, without embedding, on the freezing microtome at 16μ. The sections were mounted on slides with one per cent gelatin and prepared as follows: (a) one was stained in a one per cent solution of sudan black B in 70% alcohol for seven minutes; (b) one was stained by the carbonyl reagents of Ashbel and Seligman (1949); (c) one was stained for cholesterol by the Schultz method: (d) one was mounted unstained for study under the polarizing and fluorescence microscopes. These methods have been found useful in estimating the state of secretory activity of steroid-hormone producing tissues (Dempsey, 1948; Deane, 1951).

RESULTS AND DISCUSSION

Although occasional concentrates from both single and pooled specimens of urine proved to be toxic even in low dosage, most of the concentrates prepared after the method described above could be administered in amounts equivalent to a twelve hour output of urine without an undue loss of test animals. Results representative of those obtained with pools of moderate gonadotropic potency are presented in Table 1. It was found that a substantial increment in both testicular and prostatic weight was produced by concentrates equivalent to as little as three hours' urinary output. The seminal vesicles proved to be less sensitive to stimulation

TABLE 1. EFFECT OF AN UNFRACTIONATED EXTRACT OF HUMAN FEMALE URINE UPON THE TESTES AND ACCESSORY SEXUAL ORGANS OF HYPOPHYSECTOMIZED IMMATURE MALE RATS

Hours of urine injected	No. of rats	Mean body weight gm.	Mean and standard deviation of weight of		
			Testes mg.	Ventral prostate mg.	Seminal vesicles mg
0	7	45	135.2±25.9	5.8±1.2	6.0±1.0
3	8	49	263.3±50.4	9.8±2.2	8.8±0.9
6	9	49	310.5±20.9	12.7±2.4	9.7±1.3
9	10	53	324.4±21.6	18.8±3.6	11.1±1.5
12	9	49	336.7±32.0	18.6±3.6	10.2±2.4

than was the ventral prostate, a result in agreement with many previous reports.

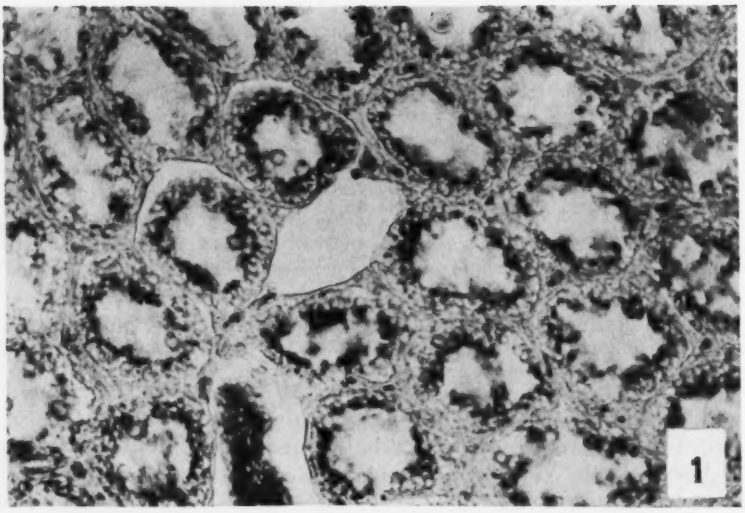
The presence of FSH in the urine concentrates is believed to account for the greater part of the increase observed in the weight of the testes. ICSH contributed to testicular growth, but to a lesser extent; hypertrophy of the Leydig cells, and tubular enlargement in response to androgen secreted by them, probably account for a small proportion of the increase in testicular weight.

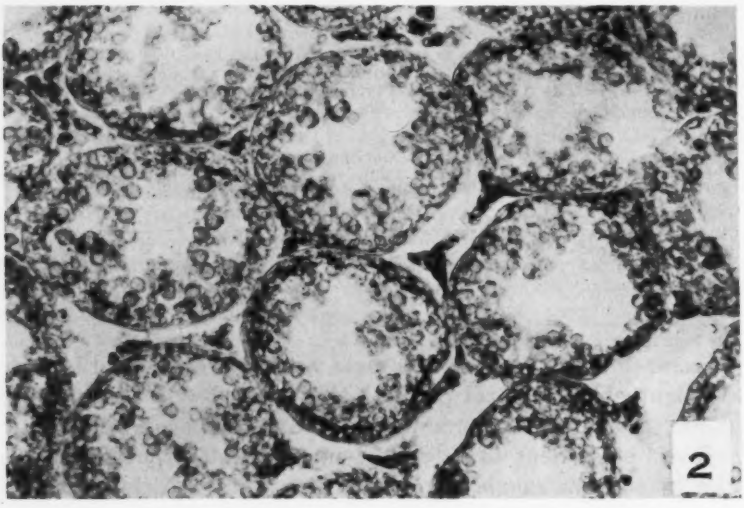
TABLE 2. FAILURE OF AN UNFRACTIONATED EXTRACT OF HUMAN FEMALE URINE TO STIMULATE GROWTH OF THE VENTRAL LOBE OF THE PROSTATE GLAND OF CASTRATED HYPOPHYSECTOMIZED RATS

Type of animal	No. of animals	Hours of urine injected	Mean and standard deviation of weight of	
			Ventral prostate, mg.	Testes, mg.
Hypophysectomized	7	0	5.8±1.2	135.2±25.9
Hypophysectomized	10	9	22.2±4.5	295.0±31.3
Hypophysectomized and castrated	10	9	6.8±1.7	—

The presence of ICSH in the extracts was manifested by the increase in the weight of the ventral prostate and seminal vesicles, which grew in response to androgen secreted by the stimulated Leydig cells. Between dosage levels equivalent to three and nine hours' output of urine, the prostatic weight was roughly proportional to the logarithm of the dose administered (Table 1).

Histological and histochemical evidence of FSH and ICSH activity was also obtained. Tubular enlargement and differentiation of the spermatocytes occurred, and the nuclei of the Sertoli cells became recognizable. The interstitial cells of the control animals resembled fibrocytes, and their meager cytoplasm was devoid of lipids (Fig. 1); those of the treated animals exhibited a more abundant cytoplasm, which contained clusters of minute lipid droplets (Fig. 2). These droplets were intensively reactive to the





Testes of rats fixed in 10% formalin, sectioned on the freezing microtome, stained with sudan black B for seven minutes, photographed with a blue filter. ×200.

FIG. 1. Testis of a control hypophysectomized rat. The tubules are small and the interstitial tissue shrunken. Fat droplets are present in the walls of the tubules, but none occur in the interstitial cells.

FIG. 2. Testis of a hypophysectomized rat injected with a urine concentrate for four days. Both the tubules and the interstitial cells are conspicuously enlarged. As compared with the control, less fat occurs in the tubules, but minute fat droplets now appear in the interstitial cells. These droplets also displayed the Ashbel-Seligman reaction for carbonyl groups and a yellowish fluorescence; the droplets of occasional cells gave the Schultz reaction for cholesterol.

carbonyl test and displayed a yellowish fluorescence. In occasional groups of cells the droplets were Schultz-positive. The small size of the droplets in the stimulated interstitial cells, their reactivity to the carbonyl test, and their fluorescence suggest that they may represent the secretory product of the Leydig cells. The larger lipid droplets observed in the tubular cells of both the control and treated rats appear not to be associated with secretory activity.

It was anticipated that the alcoholic precipitation of gonadotropins at the conclusion of the extraction process would free the protein precipitate from steroid compounds. However, in order to demonstrate that the prostatic enlargement of the treated animals was not ascribable to androgenic steroids carried through the concentration procedure, the response of hypophysectomized-castrated rats to a potent extract was compared with that of animals subjected only to hypophysectomy. The inability of the extract to stimulate prostatic growth in castrated-hypophysectomized rats can be seen from the results summarized in Table 2. Despite slight edema resulting from the castration procedure, the prostatic weights of the doubly operated animals did not differ significantly from those of the controls.

SUMMARY

A method for the preparation and identification of pituitary ICSH in human urine is described. Concentrates of as little as three hours' output of female urine were found to produce an appreciable increment in the weight of the ventral prostate of the hypophysectomized rat, the indicator of ICSH effect. Histological and histochemical evidence of ICSH activity in the urinary concentrates was also obtained. Extension of these data to permit their expression in terms of rat units of ICSH is being undertaken.

Acknowledgement

The author is indebted to Dr. Alexander Albert for generously providing facilities for this investigation at its inception in the Hormone Assay Laboratory of the Mayo Clinic, to Dr. Nathan B. Talbot for constant encouragement and support, to Dr. Ronald C. Sniffen for the histological studies, to Dr. Helen Wendler Deane for the histochemical studies and photomicrographs, to Miss Ann Bonar and Mrs. Annette C. Saltzman for expert technical assistance, and to Dr. Roy O. Greep for many helpful suggestions during the course of the study and the preparation of the manuscript.

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ADRENAL LIPID AND ALKALINE PHOSPHATASE IN HAMSTERS INFECTED WITH LEISHMANIA DONOVANI

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CHANGES in adrenal size (Selye, 1936; Tepperman, Engel and Long, 1942; Chan, 1950) sudanophilic lipid (Selye, 1936; Zwemer, 1936; Knouff, Brown and Schneider, 1941; Dosne and Dalton, 1941; Darrow and Sarason, 1944) cholesterol (Popjack, 1944; Levin, 1945; Ludewig and Chanutin, 1947; Fortier *et al.*, 1950) and ascorbic acid (Sayers and Sayers, 1948) are induced by stress. In this regard, chronic infections may serve as a continuous stress and thus influence the functional activity of the adrenal cortex. In general, adrenal hypertrophy and a loss in lipid accompanies prolonged infections (Deanesly, 1931; Menten and Smith, 1936). More recently, Sayers and his co-workers (1944) have shown that infectious diseases will deplete the adrenal gland of cholesterol and may favor adrenal exhaustion (Long, 1947; Pinchot, Close and Long, 1949).

Attention was drawn to the possible involvement of the adrenal gland in the death of Golden hamsters (*Mesocricetus auratus*) infected with *Leishmania donovani* as many hamsters exhibited marked edema. In Meleney's classical description of the histopathology of visceral leishmaniasis in the Chinese hamster (*Cricetulus griseus*) leishmania were found in clasmatocytes and even in the parenchymal cells of the adrenal. Meleney (1925) makes no mention, however, of the edema or of the other abnormalities to be reported, although Goodwin (1945) and Gellhorn *et al.* (1946) discuss the edema and Gellhorn *et al.* (1946) report amyloid in the adrenal cortex. Furthermore, Brahmachari (1942) noted a hyperpigmentation of the skin in man following leishmania infection which he related to adrenal insufficiency. The hamster presented something of a unique study in stress for unlike most mammals with lipid rich adrenal cortices the hamster adrenal is essentially free of histologically demonstrable lipid or cholesterol (Peczenik, 1944, Koneff, Simpson and Evans, 1946; Wexler, 1951) although a tinge of phospholipid and carbonyl lipid

Received for publication November 6, 1951.

This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

has been detected (Alpert, 1950; Wexler, 1951). Other components of the hamster adrenal included ascorbic acid (Alpert, 1950; Wexler, 1951) and histologically determined alkaline phosphatase enzyme (Alpert, 1950).

The current study was prompted by the paucity of information on the hamster adrenal and on the relationship of parasitic infection as a form of stress to adrenal function.

MATERIALS AND METHODS

Male golden hamsters (young adults) were obtained from a commercial breeder and maintained on Purina dog chow pellets. Infection was produced by the intraperitioneal inoculation of a suspension of ground heavily infected hamster spleen in physiological saline. The Khartoum strain of *Leishmania donovani* was used. Infected hamsters were killed by gassing and bleeding from the heart before it stopped beating at intervals of 1, 2, 4, 6 and 8 weeks to permit comparison of the adrenal glands with those from non-infected controls. One adrenal was fixed in 10% neutral formalin for 48 hours, embedded in gelatin (Zwemer, 1933) and returned to formalin until sectioned. These glands were sectioned at 15 microns on a freezing microtome, stained with sudan IV (Herxheimer's solution, Cowdry, 1943), sudan black B and nile blue sulfate and mounted in glychrogel (Zwemer, 1933). Sections were placed in 2.5% ferric ammonium sulfate at 37° C. for 3 days and then treated with sulfuric acid (Schultz, 1924) in an attempt to reveal cholesterol and its esters. The other adrenal was fixed in cold 80% alcohol, paraffin embedded and sectioned at 6 microns. The Gomori (1939, 1941) technic was used for estimation of alkaline phosphatase enzyme with the addition of 2% magnesium sulfate to augment the stain. The tissues were incubated at 37° C. with sodium B glycerophosphate for 3 and 24 hours at pH 9.3–9.4. Some adrenal sections were stained with hematoxylin and eosin and the kidney, seminal vesicle and testis were examined for alkaline phosphatase in some cases.

Intensity of infection was observed grossly by the amount of splenomegaly. This was confirmed by counts of the parasites on Giemsa-stained impression smears from a cut face of the spleen. The density was expressed as the number of leishmania per spleen cell nucleus (Goodwin, 1945). Counts above 10 Leishmania per spleen cell nucleus, as occurred in the 8th week of the infections in this series, are considered very heavy infections.

RESULTS

Adrenal gland of normal hamsters: the hamster adrenal presents the usual adrenal cortical zonations. The zone glomerulosa under the connective tissue capsule is relatively narrow and contains cells somewhat smaller than in the adjacent fasciculata. These cells may be flattened and piled in rows of 5 or 6 cells extending from capsule to fasciculata. The zona fasciculata is in palisade arrangement generally but these cells are not necessarily in cord arrangement. Furthermore, the cells may be irregular in shape with the nucleus situated centrally or peripherally (Figure 1). In the zona reticularis the cells may be rectangular or irregular in shape and nests of cells forming an acinar arrangement were observed. Enlarged sinusoids were common in the reticularis.

In general, the hamster adrenal failed to provide a positive reaction for lipid or cholesterol. However, in two animals a very slight reaction was noted for cholesterol and in two additional animals a slight lipid stain (sudan IV and sudan black B) was evident although no cholesterol reaction was observed. Alkaline phosphatase enzyme was observed in the nuclei and the cytoplasm of the midfasciculata and in the nuclei of the other zones after 3 hours' incubation (Figure 3). Increasing the incubation time to 24 hours did not alter the distribution of the enzyme but increased the intensity of the reaction and favored a slight nuclear reaction in the medulla (Figure 4).

Adrenal gland of infected hamsters: the adrenal glands from infected animals simulated those of the controls for the first two weeks. After four weeks, however, the adrenals from hamsters with spleens thrice normal size and with parasite counts at approximately 0.2 leishmania per spleen cell nucleus exhibited a beginning cellular enlargement in the reticularis with a slight loss in cytoplasmic eosinophilia (Figure 2). A tinge of lipid stain (Figure 7) and a mild but definite cholesterol reaction could also be observed in these glands. Six weeks after inoculation, the adrenals exhibited a reticularis and an inner fasciculata which contained many enlarged cells which did not stain with eosin. Other cells were of signet ring appearance, the nucleus being on the periphery and the cytoplasm appearing as a vacuole. Sections stained with sudan IV and sudan black B indicated the presence of lipid in the reticularis and fasciculata (Figure 8). Individual cell staining was variable and the lipid appeared as either fine or large cytoplasmic droplets. Some cells resembled typical fat cells with the nucleus on the periphery and a positive cytoplasmic stain but not all cells with a peripheral nucleus and large vacuolated cytoplasm stained for lipid suggesting an hydropic degeneration. A few reticular cells exhibited a rose colored reaction for unsaturated glycerides following nile blue sulfate stain and acid treatment. A strong cholesterol response was obtained in the fasciculata and reticularis. It was apparent that the degree of infection would influence the results and indeed adrenal morphology could be correlated with the degree of infection at six weeks, hamsters with few parasites failing completely to exhibit adrenal changes. The heavily infected hamsters that survived 53 to 56 days exhibited an enhancement of the morphological changes just described with increased lipid and cholesterol throughout the reticularis and fasciculata and an enlarged area of cells showing a loss in eosinophilia and apparent hydropic degeneration. The reaction for unsaturated glycerides was more widespread.

Alkaline phosphatase enzyme concentration and distribution was

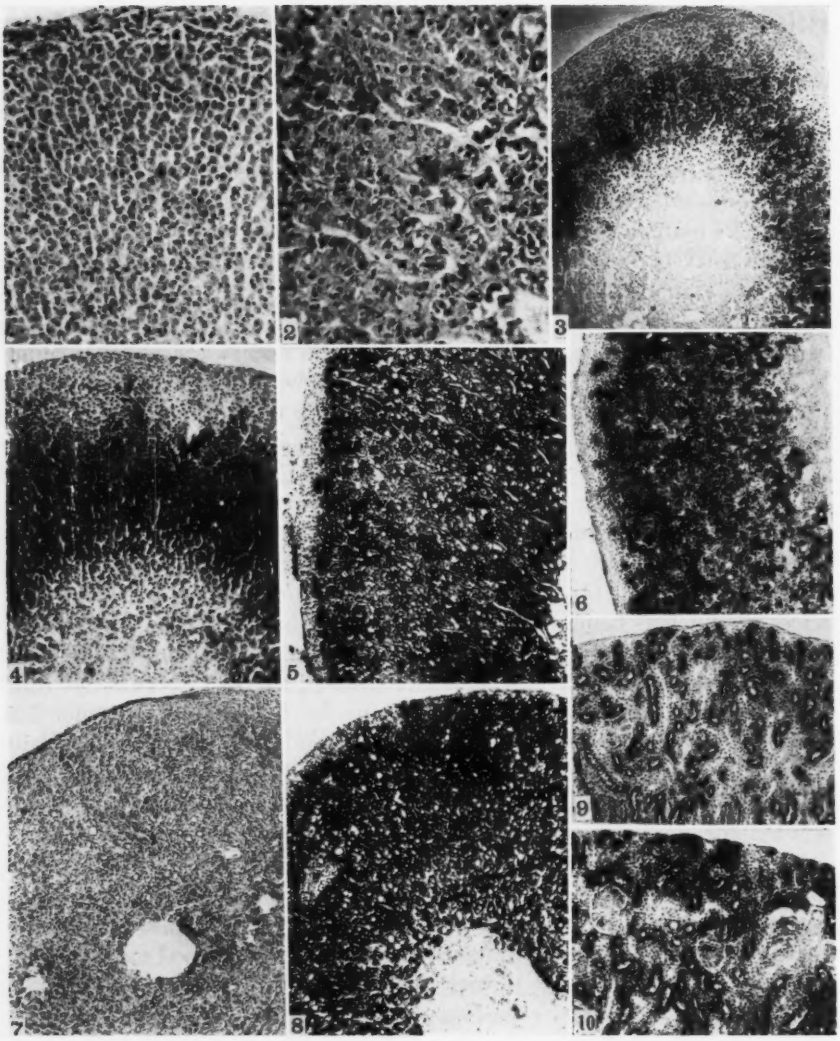

PLATE I

FIG. 1. Normal hamster adrenal cortex. Irregularity of cell size and nuclear position shown. H & E.

FIG. 2. Reticularis of hamster adrenal. Cells enlarged and eosinophilia lacking.

FIG. 3. Alkaline phosphatase. 3 hour incubation.

FIG. 4. Alkaline phosphatase. 24 hour incubation. Note increased stain intensity.

FIG. 5. Alkaline phosphatase in hamster adrenal cortex 56 days after infecting with

sharply influenced by parasitic infection beginning with the fourth week. In general, the enzyme concentration after 3 hours incubation was maximal and as intense as that seen in fascicular cell cytoplasm of control adrenals after 24 hours' incubation. The enzyme was essentially cytoplasmic and had distributed itself throughout all of the reticularis and fasciculata stopping as if at a boundary at the glomerulosa which appeared unchanged (Figure 5). The areas of cells which failed to stain for lipid despite their fat cell appearance did not react to alkaline phosphatase in the cytoplasm. An intermediate degree of reaction provided an adrenal in which only varied groups of cells would exhibit an increased intensity of phosphatase reaction (Figure 6). The altered enzyme reaction occurred only in those glands in which lipid and cholesterol were also detected.

Distribution of alkaline phosphatase in the kidney was the same as described for other species. The nuclei of proximal and distal convoluted tubules were most prominent but the brush border of the proximal convoluted tubules contained a marked concentration of the enzyme (Figure 9). Kidneys from infected hamsters seemed to have a slightly greater enzyme concentration (Figure 10). In the testis, alkaline phosphatase was present in the basement membrane and in spermatogonial nuclei. In the seminal vesicles an intense subepithelial stain was observed in the three normal hamsters studied.

DISCUSSION

The normal hamster adrenal gland was found to be lipid poor in agreement with previous investigators (Peczenik, 1944; Koneff, Simpson and Evans, 1946; Alpert, 1950, Wexler, 1951). The occasional presence of a tinge of lipid may represent individual variations or may be related to the seasonal cycle which Kayser and Aron (1950) find to occur in the hamster adrenal cortex. Accumulation of lipid can be induced by stilbestrol administration (Peczenik, 1944; Koneff, Simpson and Evans, 1946; Alpert, 1950). Alpert noted, however, that the sudanophilic lipid following estrogen was due to degeneration pigments or lipochromes in all but one case, and also that ACTH did not influence adrenal lipid. Our studies

Leishmania. 3 hour incubation.

FIG. 6. Alkaline phosphatase in hamster adrenal cortex 42 days after infecting with Leishmania. 3 hour incubation.

FIG. 7. Adrenal cortex of a hamster infected for 28 days. Sudan black B stains very slightly.

FIG. 8. Marked accumulation of lipid in adrenal cortex in hamster infected for 42 days. Sudan black B. Note increased concentration and distribution.

Fig. 9. Alkaline phosphatase in normal hamster kidney. 3 hour incubation.

FIG. 10. Alkaline phosphatase in an infected hamster kidney. 3 hour incubation.

reveal that cholesterol and lipid will accumulate in the adrenal of hamsters infected with leishmaniasis and have considered this a response to stress. One might anticipate from our results that ACTH would stimulate the adrenal changes induced by our stress conditions and indeed ACTH might do so for it should be noted that Alpert (1950) injected ACTH for only 12 days whereas our initial adrenal alterations were seen in four weeks. On the other hand, Fortier *et al.* (1950) have shown that the adrenal response to repeated ACTH administration and to stress may differ.

Leishmania infection results in adrenal cortical changes which are suggestive of exhaustion. Although the accumulation of cholesterol and lipid could represent the assimilation of materials to be used for cortical hormone formation it may also signify the gland's inability to use these substances, for in the later stages of infection many cortical cells apparently are undergoing hydropic degeneration. Peczenik (1944) observed large non-sudanophilic vacuoles in cortical cells in hamsters receiving stilbestrol. In contrast to the lipid poor hamster adrenal, the lipid rich rat adrenal contains less lipid after prolonged infection (Deanesley, 1931; Menten and Smith, 1936) although Andersen (1935) mentions that adrenals from rats with spontaneous infections have an increased lipid content. Recently, Pinchot, Close and Long (1949) in studying tularemia infection in the rat noted a low adrenal cholesterol content at a time when ascorbic acid levels were above normal and adrenal insufficiency due to adrenal exhaustion was suggested as a contributing cause of death. Apparently the immediate tendency for the rat adrenal to lose lipid, cholesterol and ascorbic acid following stress or ACTH is not maintained when continued stress is studied (Fortier *et al.*, 1950). Furthermore, sudanophilia may be dissociated from cholesterol and ascorbic acid levels as neutral fat (Popjack, 1944) and phospholipid (Knouff, Brown and Schneider, 1941; Popjack, 1944; Ludewig and Chanutin, 1947) may remain unaltered while cholesterol drops. In our studies, unlike those in the rat, infection increases sudanophilic lipid and cholesterol.

Herlant and Timiras (1950) explored varied rat tissues for changes in alkaline phosphatase concentration induced by stress and observed an increase in liver, lung, spleen, lymph nodes and thymus but no change in the kidney. In the hamster, stress induced by infection may have increased alkaline phosphatase in the kidney slightly but the profound change was seen in the adrenal glands where concentration was increased and the distribution more widespread. No explanation for this change is apparent but it might be related to phospholipid metabolism.

SUMMARY

Leishmania infection in hamsters was followed in 4 weeks by an accumu-

lation of lipid and cholesterol in the adrenal cortex normally devoid of these substances. The response was related to the degree of infection. Prolonged stress induced by Leishmania infection alters the morphology of the adrenal cortex sufficiently to suggest adrenal exhaustion as a contributing cause to the death of the animals.

The mid-fascicular cells of the normal hamster adrenal contained cytoplasmic alkaline phosphatase. Following infection, the enzyme concentration increased in the adrenal cortex and was distributed in the cytoplasm of the entire fasciculata and reticularis.

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BASAL OXYGEN CONSUMPTION OF HEREDITARILY OBESE AND DIABETIC MICE

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PREVIOUS publications have described certain metabolic characteristics of a strain of mice (Obob) presenting a high proportion of hereditarily obese individuals. The obesity and associated abnormalities appears to be a simple Mendelian recessive trait. It has been shown (Mayer *et al.*, 1951) that, when allowed a free choice of nutrients, obese individuals ate 25 per cent more calories than the non-obese siblings; the proportion of calories derived from fat, protein and carbohydrate differed markedly between obese and non-obese animals, with the obese selecting proportionately far less carbohydrate, less protein and almost twice as much fat than did the non-obese. The obese animals were found to exhibit diabetes mellitus (Mayer, Bates, and Dickie, 1951), with blood sugar levels of fed individuals ranging up to 350 mg. per cent; the glucosuria is of the order of 3 g. per cent. Furthermore, the obese animals present a striking insulin resistance, doses of 400 I.U. of insulin per Kg. leaving them unaffected. Histopathological studies of the obese animals (Bleisch, Mayer and Dickie, in press) revealed centripetal fat deposition, cutaneous atrophy, non-healing ulcers of the skin and hyperplasia of the islets of Langerhans. Structural changes of endocrine organs (other than the pancreas) were not demonstrated. Lesions of the hypothalamus, a region of special interest because of its relation to the regulation of food intake, were not detected either. Obese individuals had low hepatic glycogen stores, while their non-obese siblings present consistently higher amounts than did Swiss and CBL/57 mice examined in the same conditions (Bleisch, Mayer, and Dickie, in press).

In the course of the continued search for the primary inherited metabolic

Received for publication November 19, 1951.

[1] The portion of the work done in the Department of Nutrition, Harvard School of Public Health, was supported in part by grants-in-aid from the National Heart Institute, of the National Institutes of Health, USPHS; The Milbank Memorial Fund, New York; and the Nutrition Foundation, Inc., New York. The portion of the work done at the Roscoe B. Jackson Memorial Laboratory has been aided by a grant from the National Cancer Institute.

abnormality, a study was undertaken of the respiratory metabolism of the obese and non-obese animals. Determinations of oxygen consumption and respiratory quotients in various conditions were obtained. This report is essentially concerned with the fasting ("basal") oxygen consumption of obese and non-obese animals, with indications of the effect of thyroxine injection on weights and respiratory metabolism.

MATERIALS AND METHODS

A total of 19 animals were used in the first series of experiments (determination of basal oxygen consumption). They fell into three groups: 6 obese and 7 non-obese siblings of the Obob strain and, for purpose of comparison, a group of 6 brown controls. In the second series of experiments (effects of thyroxine) 8 animals, 4 obese and 4 non-obese were used. The weights of the obese mice ranged from 42.6 to 60.0 g.; that of the non-obese siblings from 25.4 to 30.1 g.; and that of the brown mice from 21.5 to 31.3 g. except for one animal which weighed 39.9 g. The age of the animals varied between 3 months and 1 year. The animals were housed in individual cages in a constant temperature room. They were fed ad libitum water and dog pellets. They were fasted for 16 hours prior to each determination.

Two open-circuit trains were used for the determinations. As the amounts of water and vapor and carbon dioxide given off by mice in the space of an hour are very small, it is essential to operate under conditions where fixation of these gases is total both before and after the respiratory chamber. Consequently it was found that very elaborate absorbing trains were required in order to avoid any difference in the weight of the check tubes before and after the determination. Soda lime was used to absorb carbon dioxide; "drierite" (anhydrous calcium sulfate), to absorb water. The latter was colored by an indicator which changed from deep blue to lavender to pink with hydration. It was found necessary to change the reagents in the absorbing towers every day and in the U-tubes before every run. The water-absorbing units were entirely filled with drierite. The carbon dioxide absorbing units were filled (going from the inlet to the outlet), four-fifths with soda lime and one-fifth with drierite. The function of the drierite in these tubes is to absorb the water formed in the combination of carbon dioxide with the fixed bases. Failure to absorb this water completely results in low carbon dioxide values. Each train consisted of fifteen pieces divided in three sections. The first section was designed to free the incoming air of carbon dioxide and water and comprised: an inlet, made of a calcium chloride tube filled with drierite; a sulfuric acid tower filled with concentrated acid and with a "fine" bubbler; one tower (drierite) and three towers (soda lime, drierite), one carbon dioxide check tube and one water check tube. Then followed the respiratory chamber, made of a short 130 cc. wide-mouthed bottle, a volume found to be large enough to prevent the mouse from struggling and provide a small reserve of air as cushion against possible fluctuations, yet small enough to discourage physical activity and minimize dead space. A three-hole stopper was used with one hole for the inlet, one for the outlet and one for a thermometer. The absorbing train proper was made of two drierite U-tubes, the second being the water check tube; and three soda lime-drierite tubes, the last one being the carbon dioxide check tube. A manometer was attached to the train before the outlet (to the vacuum tap). All joints were made of plastic tubing. The temperature outside of the train was maintained within narrow limits. The temperature within the chamber did not rise by more than 2° C. during operation. A depression

of 3 cm. of mercury was found optimal. All animals were made to urinate (by gentle pressure on the abdomen) before the experiment. If any animal urinated *during* the experiment, the first water-absorbing tube immediately changed color and the determination was discontinued. All weights were obtained to the nearest mg. with a chainomatic balance graduated in tenths of mg. Rapid weighing, particularly of the animal in the respiratory chamber is essential. Oxygen consumption is obtained as the difference between weights of water plus carbon dioxide given off and weight loss of the respiratory chamber containing the animal.

In the second series of experiments, the animals, obese and non-obese, were injected daily for two weeks with 50 μg. of thyroxin (crystals, Squibb, dissolved in sterile water). Basal oxygen consumption was followed. "Fed" blood glucose values were obtained before and after the period of treatment. Insulin resistance tests were conducted in the obese animals by injection of 10 I.U. insulin (Iletin, Lilly) in the fasting state.

RESULTS

For each animal, at least three concordant low determinations were obtained and averaged. The results recorded in Table 1 represent the means of individual averages. They are expressed in terms of volume of oxygen at 0°C and normal pressure. Generally speaking, two or three higher values were recorded before lower values, in good agreement with

TABLE 1. BASAL OXYGEN CONSUMPTION OF OBESE AND NON-OBESE MICE OF THE OBOB STRAIN AND OF BROWN MICE

	Average weight (g.)	Average total oxygen consumption (cc./min.)	Average oxygen consumption per g. (cc./min./g.)	Average oxygen consumption per unit surface (cc./min./g.$^{0.7}$)
Obese	53.7 ± 9.1	1.398 ± 0.30	0.0277 ± .001	0.098 ± 0.003
Non-obese	28.2 ± 1.9	1.678 ± 0.25	0.0616 ± .012	0.175 ± 0.031
Brown	29.5 ± 7.0	1.236 ± 0.48	0.0404 ± .013	0.106 ± 0.018

(Figures after ± signs represent standard deviations.)

one another, were reached. This no doubt corresponds to the acclimatization of the animals to the experimental procedure.

Table 1 summarizes the results obtained with the untreated obese and non-obese siblings. Results obtained on the brown mice are included for comparative purposes. It is readily seen that, on a weight basis, the oxygen consumption of the obese animals is less than 50% than that of the non-obese ($P < 0.001$). Expressing the results on the basis of surface area, and taking the weight to the 0.7 power to be proportional to the body surface (Brody, 1944), one finds that the "basal metabolic rate" of the obese animal is 44% lower than that of the obese animals ($P < 0.001$). In spite of a body weight almost double of that of the non-obese animals, the total oxygen consumption of the obese mice is thus somewhat (though not significantly) smaller than that of the obese animals. Respiratory quotients for both obese and non-obese animals varied between 0.70 and

0.74 with an average of 0.72; no significant difference thus appeared in the respiratory quotients under "basal" conditions. A significant difference was found between the two strains Obob non-obese and brown mice.

The daily dose of 50 μg. per mouse was found to bring the basal metabolic rate of obese mice to .178 cc./g./min., of the same order of magnitude as that of the untreated mice. That of normal mice was elevated to .202 cc./g./min. Under these conditions, the obese mice were found to suffer a rapid loss of weight. For example, one obese mouse lost 18.5 g. in 14 days. Another lost 8.7 g. in 14 days. Blood sugar levels were not significantly affected and in the fed state remained of the order of 400 mg./per cent. The daily food intake of the animals dropped from 5 to 6 g. to an average of 2.9 g. Generally speaking, older obese mice (over 8 months) were found to be more affected than young obese mice (less than 4 months). By contrast, the weights of normal animals were generally not affected by a similar dose of thyroxine; only one animal lost some weight, and this only 2.7 g. in 14 days. Another non-obese animal actually gained 1 g. in 14 days on the same treatment. It may be remarked that on a weight basis, the dose received by the non-obese (50 μg. for 28.2 g.) was higher than that received by the obese (50 μg. for 53.7 g.).

DISCUSSION

The fact that there is a difference between basal metabolic rates of normal animals of two different strains is not remarkable. Various strains of mice have been shown to exhibit striking differences in nutritional requirements and metabolism (Fenton and Cowgill, 1947).

On the other hand, the difference recorded between obese and non-obese animals is surprising. The finding that obese mice do not have a higher total oxygen consumption than non-obese animals is unexpected when considered in the light of extensive comparisons between obese and non-obese human subjects (Boothby and Sandiford, 1922; Strouse, Wang and Dye, 1924; Grafe, 1933). In ordinary human obesity, the parallel increase in calculated body surface and oxygen consumption may be a coincidence depending on the shape of the human body and the respiratory rate of adipose tissues. In the case of these obese mice, it seems probable that adipose tissues are unusually inert, a possibility which is being tested using mass spectrometric technique on deuterium turnover.

Hypothyroidism is, of course, known to depress basal metabolism. The extreme sensitivity of the obese animals to doses of thyroxine which leave non-obese animals almost unaffected, in spite of their very much smaller body weight might suggest hypothyroidism. The fact that the obese animals decrease their food intake when given thyroxine and seem

to become able to catabolize their fat reserves, also seems to point to hypofunction of the thyroid. The relative hypercholesteremia of obese mice (total cholesterol values of about 200 mg. per cent as compared to 100 mg. per cent for the non-obese) would be strongly suggestive of hypothyroidism if it were not for the fact that concomitant diabetes, complicates the interpretation of the finding. On the other hand, no abnormality was detected in histopathological examination of the thyroid. Postulating that the primary functional lesion is thyroid hypofunction would not explain the caloric overconsumption, the obesity, the hyperglycemia and other attendant abnormalities. Hypothyroidism has never been found to be associated with insulin resistance; in fact hyperthyroidism is the condition often associated with this characteristic (Thorn and Forsham, 1950).

Animals subjected to experimental lesions of certain parts of the hypothalamus develop obesity and show a low basal metabolic rate (Brobeck, Tepperman and Long, 1943); on the other hand, the bulk of the evidence indicates that hypothalamic obesity is associated with hypersensitivity to insulin and hypoglycemia (Barris and Ingram, 1936; Davis, 1934; Davis, Cleveland and Ingram, 1935; Keller, 1939; Brobeck, 1940). It has been shown already (Bleisch, Mayer and Dickie, in press) that certain aspects of the syndrome, such as centripetal obesity, insulin resistance, diabetes and skin change recall certain aspects of classical adrenal hyperactivity in the human. On the other hand, other aspects of this syndrome argue against this possibility. They are the low liver glycogen, the extreme lability of blood glucose, the absence of any recognizable histological lesion of the pituitary or adrenals, the inability of the obese animals to subsist on a carbohydrate free diet, even if it includes a fair proportion of protein (unpublished observation), the extremely low basal metabolism, and the fact that administering ACTH to non-obese animals (unpublished observation) does not reproduce "obese" symptoms.

Because the syndrome is inherited as a simple Mendelian recessive trait, it most probably has a single etiological cause, which in turn may produce many secondary changes. The search for this primary metabolic abnormality is being pursued.[2]

SUMMARY

A study was conducted of the oxygen consumption of obese and non-

[2] "Since this paper was submitted, the primary genetic lesion has been found to be a partial block of oxidation of C_2-fragments (Guggenheim and Mayer, *J. Biol. Chem.* in press). The mechanism of the etiology of the diabetes and other secondary symptoms has also been elucidated in part (Mayer, Russell, Bates and Dickie, *Metabolism*, in press)."

obese members of the "Obob" strain of mice. The obese members of this strain were shown to present, in addition to the insulin-resistant type of diabetes and other characteristic symptoms previously described, a very low rate of fasting oxygen consumption. On a body weight basis, their oxygen consumption was less than half that of the non-obese animals, on a body surface basis, their "basal metabolic rate" was lower by more than 40%. Although their average weight was almost twice that of the non-obese animals, even the total oxygen consumption was somewhat (though not significantly) lower than that of the non-obese.

When the basal metabolic rate of the obese animals was brought up to non-obese levels by injections of thyroxine, a rapid loss of body weight ensued. The same dose given to non-obese animals (although greater in a body weight basis) had a much smaller effect on oxygen consumption and no effect on body weight. Implications of these findings are discussed.

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THE EFFECT OF CORTISONE AND CORTICOTROPIN ON THE BIOLOGIC DECAY OF THYROIDAL RADIOIODINE

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SEVERAL recent studies have been interpreted as showing that cortisone and corticotropin induce hypothyroidism by suppressing the secretion of thyrotropin by the pituitary (Hill *et al.*, 1950; Frederickson, 1951). The evidence for this conclusion has been reviewed in detail elsewhere (Albert, 1952). Part of this evidence is the observation that I^{131} uptake by the thyroid in both animals and man is diminished during administration of either corticotropin or cortisone. If thyroid function is defined in terms of the accumulation of I^{131} over a given interval (a poor definition), then it could be said that both cortisone and corticotropin reduce thyroid function. However, it would not necessarily follow that such a depression of thyroid function resulted from suppression of thyrotropin, since there are many possible explanations for the reduction of I^{131} accumulation apart from any alteration in the secretion of thyrotropin.

In evaluating studies with I^{131} the definition of "thyroid function" becomes of paramount importance. Thyroid function is perhaps best defined in terms of the amount of hormone secreted by the gland per unit of time. A direct and absolute measurement of hormonal secretion rate, therefore, would be more precise than I^{131} uptake, but this is technically not feasible at present. An indirect measure of secretion rate is available [determination of the biologic decay of thyroidal I^{131} (Albert, 1951)] and may provide more reliable and unambiguous information about thyroid function than the measurement of I^{131} accumulation. It was of interest, therefore, to ascertain whether cortisone and corticotropin would affect the biologic decay of thyroidal I^{131}. After this work had been completed, a similar study was published by Perry (1951), whose results are confirmed by our data. Because of the controversial nature of this subject and because our technic differs in several major respects from that of Perry, it was thought worth while to present our data briefly.

Received for publication November 19, 1951.

MATERIAL AND METHODS

Fifty weanling male rats (Sprague-Dawley) maintained on an iodine-deficient diet for two weeks were employed in two experiments. In the first experiment 25 rats were divided into three groups: one group remained as a control group, another group received 2 I.U. corticotropin per day for five days, and the third group received 2 mg. cortisone per day for five days. On the sixth day, 2 microcuries of carrier-free I^{131} were injected subcutaneously into all rats and *in vivo* measurements over the thyroid were made at two, four, six and twenty-four hours, and thereafter daily for fourteen days. The injections of corticotropin and cortisone were continued throughout the entire period of observation. The determinations for the twenty-four-hour uptake of I^{131} (Randall and Albert, 1951) and for the secretion rate (Albert, 1951) have been described.

In the second experiment, all 25 animals were injected with 2 microcuries I^{131} and a count for each animal was made forty-eight hours later. They were then segregated into three groups as described in the preceding paragraph, one maintained as control, and the other two groups injected with cortisone and corticotropin as outlined. Daily readings were taken for a period of two weeks. The purpose of this variation in experimental design will be given later.

RESULTS

The results for the two experiments are given in Table 1, which shows that no significant effect has been induced in the secretion rate either by

TABLE 1. EFFECT OF CORTICOTROPIN AND CORTISONE ON THE BIOLOGIC DECAY OF THYROIDAL RADIOIODINE

	Experiment 1				Experiment 2			
	Animals	Secretion rate			Animals	Secretion rate		
		Mean	Sem.*	"t"		Mean	Sem.*	"t"
Controls	8	6.97	0.60		8	9.51	0.71	
Corticotropin	8	9.16	0.82	2.19	8	7.32	0.58	1.93
Cortisone	7	8.26	0.46	1.72	9	7.87	1.29	1.29

* Standard error of mean.

cortisone or by corticotropin. The uptake of the tracer dose at twenty-four hours was, however, depressed by both substances. Cortisone lowered the uptake to an average of 42 per cent, and corticotropin to 62 per cent of the controls.

The second experiment, in which cortisone and corticotropin were administered *after* the thyroids were labeled with I^{131}, was done in view of the diminished I^{131} uptake, which presumably could signify diminished I^{127} uptake. Were this the case, then the thyroids of the experimental rats after a week's treatment with cortisone and corticotropin might have less total I^{127} than the controls. With less total thyroidal I^{127}, a secretion rate equal to that of control animals could mean that the cortisone-treated and corticotropin-treated animals actually secreted less hormone

in absolute terms than the controls with a higher thyroidal I^{127}. That this is probably not the case is indicated by the result that the secretion rates in the experimental groups were not affected whether the animals were treated before or after their thyroids were labeled with I^{131}.

DISCUSSION

That cortisone and corticotropin reduced the twenty-four-hour uptake of I^{131} is in accord with most (Perry, 1951; Money *et al.*, 1950, 1951; Soffer *et al.*, 1951), but not all (Botkin and Jensen, 1951), investigators. Such a depression might result from inhibition of thyrotropin. It could also result from an increased renal clearance of iodide, from a direct inhibitory action on the permeability of the thyroid cells or on any of the mechanisms necessary for the selective accumulation of iodide by the thyroid cells, from an increase in iodide space, or from other factors. Which of these or other possibilities is involved remains for further study, but at least one mechanism (inhibition of thyrotropin) seems to be ruled out: In the absence (Randall, Lorenz and Albert, 1951) or the inhibition (Albert and Tenney, 1951) of thyrotropin both the uptake and discharge of I^{131} are markedly lowered. If cortisone and corticotropin inhibited thyrotropin, it is difficult to imagine how the uptake could be depressed while the secretion rate was unaffected.

SUMMARY

Corticotropin and cortisone, while lowering the twenty-four-hour accumulation of I^{131}, did not affect the discharge of thyroidal I^{131}.

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THE EFFECT OF HYPOPHYSECTOMY ON THE RENAL CLEARANCE OF I^{131}

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THE amount of I^{131} collected by the thyroid is proportional to the concentration of I^{131} in the blood. Hence, if thyroid function remains at a constant level, the amount of I^{131} accumulated by the thyroid will be determined in part by factors which may influence its concentration in the blood. Such factors would include ability of the kidneys to excrete iodide, changes in blood volume, extracellular fluid volume or other alterations of the iodide space.

The thyroid of the hypophysectomized rat collects about one eighth as much radioiodine as does that of the normal animal (Randall and Albert, 1951). It is assumed that this depression denotes a diminution of thyroid function resulting from the loss of thyrotropin. However, it is conceivable that the lowered accumulation of I^{131} could be due to some extrathyroidal mechanism. Because of the marked polyuria which occurs during the first few days following hypophysectomy, it was thought worth while to determine the effect of hypophysectomy on the renal clearance of I^{131}.

MATERIAL AND METHODS

Male rats (Wistar strain) maintained on a laboratory diet of Purina fox chow were employed. Tests with radioiodine were performed five days after hypophysectomy (Randall and Albert, 1951). For the determination of renal clearance, 10 microcuries of I^{131} contained in a small volume (0.1–0.3 cc.) were injected into the external jugular vein. Preliminary studies to establish the character of the disappearance of I^{131} from the blood indicated that a diphasic curve was present. A sharp drop in blood I^{131} occurred within one hour, followed by a slower second phase extending throughout the next twenty-four to forty-eight hours. Consequently, the first blood sample was obtained one hour after injection by withdrawing 0.1 cc. of blood from the opposite external jugular vein. The time was noted, and the blood sample was delivered to a glass tube of 83 cc. capacity especially designed for a gamma ray counter (Johnson and Albert, 1951). A drop of 3% sodium hydroxide was added, and the tube was made up to mark with distilled water. The animals were then placed in individual glass vessels. the bottom of which was covered with a sheet of filter paper moistened with a solution of 1% sodium iodide and 1% sodium hydroxide. Urine was absorbed on the filter paper, being separated from feces by a wire screen. Water was allowed ad libitum, but food was

Received for publication November 19, 1951.

withdrawn during the period of time necessary to determine the renal clearance. Approximately at either five or twenty-four hours after the first blood sample, another blood sample was similarly obtained, the time being recorded. At this time, the rats were anesthetized and the bladder was cleared of residual urine, which was added to the filter paper. The filter paper was rolled up and placed in a glass tube. The glass vessel was washed with a small amount of 1% sodium iodide and 1% sodium hydroxide solution, and the washings were added to the tube containing the filter paper. The amount of I^{131} in the blood samples and in the urine was then simultaneously determined (Johnson and Albert, 1951). The renal clearance of I^{131} was calculated by the conventional formula, UV/B, where UV is the urinary counts per minute and B is the mean blood concentration. The mean blood concentration is given by:

$$\frac{(A-B)\times 0.434}{\log A - \log B}$$

where A is the number of counts per cubic centimeter of the first sample of blood, and B the counts of the second sample of blood.

RESULTS

The results are shown in Table 1. Renal clearances were determined both five and twenty-four hours after administration of I^{131}, the latter to

TABLE 1. RENAL CLEARANCE OF I^{131} (CC. $\times 10^3$ PER MIN.) IN RATS AFTER VARIOUS EXPERIMENTAL PROCEDURES

Conditions	Animals	Mean	Sem.*
Normal (5 hr.)	27	26.91	3.97
Hypophysectomized (5 hr.)	30	6.73	1.08
Normal (24 hr.)	10	36.33	3.40
Hypophysectomized (24 hr.)	16	4.43	0.92
Thyroidectomized (5 hr.)	14	25.14	4.29
Adrenalectomized (5 hr.)	5	46.30	8.43

* Standard error of mean.

insure a more accurate value. In spite of the high variation from animal to animal, reflected in the large standard error of the mean, it appears that hypophysectomy depressed the renal clearance of I^{131} to from one fourth to one ninth that of normal animals. These differences are highly significant, "t" values being 4.9 and 9.8 for the five-hour and twenty-four-hour determinations, respectively. A number of determinations were performed on rats thyroidectomized or adrenalectomized five days previously. The renal clearance of iodide was not seriously affected, as compared with controls ("t" = 0.3 and 2.0 respectively). Consequently, the depression of renal iodide clearance resulting from hypophysectomy probably cannot be ascribed to changes induced in thyroid or adrenal function by hypophysectomy.

TABLE 2. RENAL CLEARANCE OF I^{131} (CC. $\times 10^3$ PER MIN.) IN HYPOPHYSECTOMIZED RATS BEFORE AND AFTER INJECTIONS OF PITUITARY SUSPENSIONS

Before*	After
9.09	5.78
9.92	57.42
9.59	68.75
2.43	13.98
3.30	17.30
0.91	2.16
0.82	13.00
3.00	22.10
13.00	12.40
1.60	15.60

* The first 4 animals had clearance values determined over a period of five hours; the last 6, over a period of twenty-four hours.

A few experiments were performed to determine whether the normal value for the renal clearance could be restored in hypophysectomized animals. After clearances were measured in hypophysectomized rats, each animal was injected subcutaneously with a suspension of rat pituitaries, equivalent to one pituitary daily for three days. Clearance of iodide was then redetermined (Table 2). The average difference in clearance values was 17.49 ± 6.46. Analysis of the paired data gave a "t" value of 2.71, corresponding to a P/2 value of between 0.01 and 0.02.

DISCUSSION

A depression of radioiodide clearance in hypophysectomized animals would indicate that changes in renal excretion do not contribute to the depression of thyroidal collection of iodine following hypophysectomy. On the contrary, the hypophysectomized rat has a greater opportunity to collect iodine owing to the concomitantly lowered renal clearance. Furthermore, the concentration of I^{131} in blood and tissues of the hypophysectomized animal should be higher than that of normal animals over a comparable period.

An indication of the correctness of this view was obtained by observing

TABLE 3. MEAN I^{131} CONTENT (PER CENT OF DOSE) OF BODY AND EXCRETIONS OF 15 NORMAL AND 10 HYPOPHYSECTOMIZED RATS

	Time after injection, hr.				
	0	7	24	48	72
Total body I^{131}					
Normal	100	72	27	11	7
Hypophysectomized	100	82	64	44	23
Urinary and fecal I^{131}					
Normal	0	25	69	86	92
Hypophysectomized	0	9	31	53	71

the behavior of I^{131} in 15 normal and in 10 hypophysectomized rats of the Sprague-Dawley strain maintained for one week on an iodine-deficient diet. Under these conditions about 10 per cent of the dose is usually collected in the thyroid of the normal animal and not more than 1 or 2 per cent in the thyroid of the hypophysectomized animal. Immediately after intravenous injection of 1 microcurie of I^{131} the entire rat was placed in the glass tube and a count was obtained. This count represented 100 per cent of the dose. The rats were then placed in individual cages. At various intervals, a count of the entire rat was again made and the I^{131} of both urine and feces was determined. These results are shown in Table 3, which gives the I^{131} of the entire rat for both normal and hypophysectomized rats; and accumulatively, the I^{131} appearing in the urine and feces. It appears that the disappearance of I^{131} from the normal animals is more rapid than that from the hypophysectomized animal, and that the fecal and urinary excretion of I^{131} is greater in normal than in hypophysectomized rats. In spite of the greater availability of I^{131} throughout this three-day interval in the hypophysectomized animal, the thyroid of the normal rat collects some three to eight times more radioiodine than that of the hypophysectomized animal.

That the pituitary may influence the renal excretion of electrolytes like iodide is indicated by the few experiments with pituitary injections shown in Table 2. However, more intensive experiments are necessary to establish this with certainty, to determine which of the pituitary hormones, if any, is responsible for such an action, and to ascertain whether this renal defect observable in the first week after operation is adjusted later on.

SUMMARY

The renal clearance of radioiodine is markedly depressed five days after hypophysectomy.

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THE MAINTENANCE OF THE ADRENALECTOMIZED DOG FOR PROLONGED PERIODS WITHOUT RECOURSE TO HORMONAL THERAPY*

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THE study of many problems concerning the role of the adrenal cortex in the animal economy involves the maintenance of adrenalectomized animals without recourse to hormonal therapy. For such purposes the rat maintained on a high salt intake has been widely used, but in many cases, the dog is a much more suitable subject for investigation. Several investigators (Harrop, Soffer, Nicholson and Strauss, 1935; Allers and Kendall, 1937; Nilson, 1937; Cleghorn, Armstrong and Austen, 1939) have reported the maintenance of adrenalectomized dogs on a high sodium, low potassium diet after a preliminary period on hormonal therapy during the immediate post-operative period. However, this procedure which involves the forced-feeding of a specially prepared diet, is difficult to apply, and fails to maintain the animal in a uniform condition.

The successful application of intermittent peritoneal lavage for maintaining the bilaterally nephrectomized dog for prolonged periods (Grollman, Turner, and McLean, 1951) suggested the use of this method for maintaining a normal salt and water balance in the adrenalectomized animal. The procedure has proven adequate for maintaining the bilaterally adrenalectomized dog in good condition for periods exceeding a month without recourse at any time to the use of any hormonal therapy. In fact, it has proven superior in this respect to the use of desoxycorticosterone as a substitution therapy and offers a technique whereby many problems such as the pathological changes in the tissues, electrolyte disturbances, etc. induced by adrenalectomy can be studied in a manner previously not possible.

MATERIAL AND METHODS

Adult mongrel dogs of either sex were adrenalectomized by the procedure outlined previously (Grollman, 1936). The right gland was removed at the first operation under pentobarbital anesthesia; the left, after an interval of 1 to 2 weeks, with ether as the anesthetic. No special pre-operative preparations, such as deworming, were employed

Received for publication November 20, 1951.

* Aided by a grant from the Vaughn Fund.

nor were any hormonal preparations or special diets used at any time pre- or post-operatively. The diet consisted of commercially available canned "horse meat."

Following operation, a solution having the following composition was introduced through a No. 18 long hypodermic needle into the peritoneal cavity:

Sodium chloride	5.671 gms.
Sodium bicarbonate	2.101 gms.
Sodium lactate	3.137 gms.
Potassium chloride	0.336 gms.
Calcium chloride	0.222 gms.
Magnesium chloride	0.071 gms.
Glucose	1.000 gms.
Penicillin	100,000 units
Terramycin	25 mgs.
Pyrogen-free distilled water, sufficient to make 1 liter	

This solution was prepared by adding the indicated amounts of the three sodium salts and the penicillin to triple-distilled water which was then preserved in the refrigerator. To prevent precipitation of carbonate, the other salts, glucose and terramycin were added, after warming the solution to body temperature, just prior to its injection.

The solution just described contains the following ionic constituents: sodium, 150; bicarbonate, 25; chloride, 107; potassium, 4.5; calcium, 4.0; and magnesium, 1.5 meqs. per liter, which is essentially the concentration in which these ions are present in an ultrafiltrate of blood plasma.

About 50 cc. of the above-described solution per kilogram of body weight was used on the first day, but this was gradually increased to 100 cc. by the fifth post-operative day when healing of the operative site had occurred. For optimal conditions the fluid remaining in the peritoneal cavity is removed through a needle, as in the previously described method for peritoneal lavage (Grollman, Turner and McLean, 1950), after 8 to 12 hours and replaced with new solution. This procedure performed twice daily permits the attainment of relatively normal blood electrolyte levels. However, it is only necessary to introduce the peritoneal fluid once daily for maintaining the animals in good condition for extended periods. The amount of fluid remaining in the peritoneal cavity after 24 hours under these conditions is minimal and need not be withdrawn.

Blood pressures were determined by puncture of the femoral artery as described elsewhere (Grollman, 1946); blood analysis, by the procedures used in other studies from this laboratory (Grollman, Muirhead and Vanatta, 1948; Grollman, Turner and McLean, 1950).

As controlled procedures for the maintenance of the adrenalectomized dog we have utilized desoxycorticosterone, which has been most commonly used as a maintenance therapy; cortisone; and a combination of the two drugs which in clinical experience has proved most practical as a substitution therapy in adrenal cortical insufficiency.

OBSERVATIONS

In Table 1 are shown the survival periods and other pertinent data on 10 dogs receiving one intraperitoneal injection daily. These experiments were performed during the summer months (May to September) when the environmental temperature varied between 27 and 40° C. Control untreated animals under these conditions survive on an average for only 1 to 3 days and even under most favorable conditions the adrenalectomized untreated dog survives rarely for longer than a week (Grollman, 1936).

As noted in the last column of Table 1, a single daily injection suffices to maintain a sufficiently normal water and electrolyte balance to permit survival of the dog for prolonged periods after adrenalectomy without recourse to hormonal therapy. The animals maintained in this manner appeared relatively normal on superficial examination. The operative wounds healed promptly and the animals' appetite and general condition were excellent except that they were less active than the normal dog or one maintained on hormonal (aqueous extract) therapy.

TABLE 1. PERIOD OF SURVIVAL AND OTHER PERTINENT DATA ON 10 BILATERALLY ADRENALECTOMIZED DOGS INJECTED INTRAPERITONEALLY ONCE DAILY

Dog no.	Body weight		Average pulse rate	Average mean blood pressure	Survival
	Initially	Terminally			
	kgs.	kgs.	per min	mm. Hg	days
1	9.1	8.8	93	97	35*
2	8.0	7.9	100	112	22
3	8.4	8.0	96	95	25*
4	8.2	7.9	90	112	26
5	6.3	6.0	84	102	35*
6	6.8	6.6	94	94	34*
7	7.3	7.1	92	78	35
8	6.6	6.1	100	84	35
9	4.5	4.3	98	87	45*
10	5.0	4.6	95	90	47

* Sacrificed.

The paralysis of the extremities and hind leg weakness so characteristic of the untreated adrenalectomized dog was not observed in our animals and is also apparently induced by salt deficiency since a similar condition is seen in animals depleted of sodium and water by administration of a salt-free diet and treated with theobromine sodium salicylate (Grünwald, 1909).

The mean arterial blood pressure and pulse rates as noted in Table 1 are not greatly altered from the normal, the blood pressure being only about 10 mms. less than that observed in the well-trained resting dog. The hypotension characteristic of cortical insufficiency is thus also due to loss of extracellular fluid volume secondary to sodium depletion. The relatively slight loss of body weight observed and the absence of anorexia also indicate that these are secondary to sodium depletion.

The general impression derived from the literature would indicate that desoxycorticosterone alone is a satisfactory replacement therapy in the adrenalectomized dog; this has not been the universal experience and the use of this substance alone has proved to be inadequate unless very special precautions are taken to avoid undue stresses. Under the conditions of the present experiments, it was not possible to maintain the adrenalecto-

mized dog on desoxycorticosterone therapy (0.5 mgm. per kilo daily) for periods beyond 12 days, most of the animals succumbing during the first week post-operatively. The use of a single daily intraperitoneal injection was, accordingly, under the conditions of our experiments superior to the use of desoxycorticosterone as a means of maintaining the adrenalectomized dog.

In view of the clinical observation of the effectiveness of cortisone and of a combination of cortisone and desoxycorticosterone as a replacement therapy in Addison's disease, the capacity of these drugs to maintain the

TABLE 2. BLOOD CHEMICAL VALUES IN FASTING ADRENALECTOMIZED DOGS MAINTAINED BY TWO INTRA-PERITONEAL INJECTIONS DAILY

Dog no.	Days following adrenalectomy	Glucose	Non-protein-nitrogen	Sodium	Potassium	Chloride	CO_2 Combining power
		mgs. per cent		meq. per liter			
1	10	102	26	128	4.8	110	24
	40	71	25	130	4.0	102	25
2	15	80	24	127	5.0	102	26
	30	38	19	128	3.5	110	24
3	30	96	14	134	5.2	103	25
4	30	45	21	138	5.5	112	24
5	14	104	27	138	5.0	104	25
	35	82	18	130	5.8	107	23

adrenalectomized dog was also determined. Cortisone, alone, in doses of 0.5 mgm. per kilo of body weight, proved inadequate as a replacement therapy, the animals manifesting signs of adrenal cortical insufficiency (weakness, anorexia, and diarrhea) and succumbing in 7 to 14 days following adrenalectomy. On the other hand, a combination of desoxycorticosterone (in doses of 0.1 mgm. per kilo) and cortisone (0.5 mgm. per kilo) adequately maintained the animals in good condition.

The effectiveness of peritoneal injections and of a combination of desoxycorticosterone and cortisone therapy in maintaining the water and electrolyte balance is shown in Tables 2 and 3 in which typical data on the sodium, potassium, chloride, non-protein-nitrogen, carbon dioxide combining-power and glucose contents of the blood are recorded. A single daily intraperitoneal injection resulted in lower values for sodium, and higher values for potassium and non-protein-nitrogen than those shown in Table 2. However, even with two daily injections, as shown previously (Turner and Grollman, 1951), and as is seen in Table 2, there is a tendency for the sodium to be lower than normal and for the potassium at times to be higher than normal. Apparently, absorption from the reservoir in the

peritoneal cavity, does not keep pace with the accelerated loss of sodium through the kidney.

The fasting glucose levels of the adrenalectomized dog maintained by intraperitoneal injections, as seen in Table 2, are frequently lower than normal. This is contrary to the results obtained in acute adrenal cortical insufficiency where, as shown by Parkins, Hays, and Swingle (1936), there is no tendency for hypoglycemia to occur. The prolonged deprivation of hormone as in the experiments of Table 2 apparently results in metabolic alterations which are not observed in more acute experiments.

TABLE 3. BLOOD CHEMICAL VALUES IN ADRENALECTOMIZED DOGS MAINTAINED ON CORTISONE AND DESOXYCORTICOSTERONE THERAPY

Dog no.	Days following adrenalectomy	Glucose	Non-protein-nitrogen	Sodium	Potassium	Chloride	CO_2 Combining power
		mgs. per cent		meq. per liter			
1	10	88	19	132	3.4	110	23
2	20	120	20	130	3.2	113	22
3	10	94	24	128	3.7	101	24
4	30	76	18	130	3.4	114	24

As shown in Table 3, the daily administration of a combination of cortisone and desoxycorticosterone, results in blood chemical values which are comparable to those attained by intraperitoneal injections except for more normal glucose levels, lower levels of potassium, and higher levels of chloride.

DISCUSSION

It is generally known that any procedure which tends to correct the hyponatremia, hyperpotassemia and dehydration characteristic of adrenal cortical insufficiency will prolong the life and mitigate many of the symptoms induced by this condition. The procedure described in the present paper offers a relatively simple method whereby the defect in salt and water metabolism which results from adrenalectomy is so well corrected that the animals survive for prolonged periods and even tolerate the severe strain of anesthesia and abnormal environmental conditions without the use of hormone. It must be concluded that the loss of salt and water which follows adrenalectomy is the critical factor which in the dog results in the characteristic symptoms observed following this operation. The marked hypotension and circulatory insufficiency secondary to sodium depletion is apparently responsible for most of the acute symptoms of adrenal cortical insufficiency including the hind-leg weakness, anorexia, and vomiting. Loss of the hormone results, however, in the chronic affects

of adrenal cortical insufficiency and although prevention of salt depletion permits prolonged survival, it will not permit "indefinite" survival as claimed by earlier workers. Following the first demonstration of the beneficent effects of salt administration in Addison's disease, it was claimed that maintenance of a normal salt and water metabolism alone would suffice to maintain life in the face of adrenocortical insufficiency. Clinical experience soon demonstrated the inadequacy of such therapy.

The procedure outlined offers a method for studying the factors involved in adrenal cortical insufficiency uncomplicated by abnormalities in salt and water metabolism. The changes induced by this hormonal deficiency as observed by histological and analytical studies of the tissues will be the subject of future reports. The intraperitoneal route of administration as described may also be used with advantage in patients suffering from Addison's disease in crisis or in other conditions in which depletion of the extra-cellular-fluid requires correction.

SUMMARY

A procedure is described for maintaining the bilaterally adrenalectomized dog alive in good condition for prolonged periods without recourse to the use of hormones or special diets pre- or post-operatively. The method consists of injecting intraperitoneally once or twice daily a solution containing the inorganic constituents of the blood in the concentration in which they are normally present in plasma. By this simple procedure the water and electrolyte pattern of the blood is maintained relatively normal thus preventing the appearance of those symptoms of acute adrenal cortical insufficiency which are secondary to sodium depletion and oligemia. The use of desoxycorticosterone, cortisone, and a combination of these two drugs as replacement therapy in the adrenalectomized dog were also studied.

Acknowledgment

The terramycin used in the present study was kindly furnished by Pfizer and Co. through the courtesy of Dr. W. Alan Wright; the desoxycorticosterone by the Schering Corp., through the courtesy of Dr. L. H. Hemingway; and the cortisone, by Merck and Co., through the courtesy of Dr. Leo V. Curtin. Messrs. H. L. Harle, G. L. Marable, J. M. Mims, W. M. Ott, and F. C. Rector rendered valuable technical assistance in the present study.

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HISTOCHEMICAL DEMONSTRATION OF KETOSTEROIDS IN ADRENAL CORTICAL TUMORS WITH OR WITHOUT AN ASSOCIATED CUSHING'S SYNDROME[1]

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A HISTOCHEMICAL method for demonstrating carbonyl groups in frozen sections of formalin-fixed tissue by the formation of a blue azo dye in reactive sites has been described (Seligman and Ashbel, 1949; Ashbel and Seligman, 1949, and Seligman, Friedman and Herz 1949). This method also demonstrates carbonyl groups in a poorly soluble proteolipid-like substance in myelin (Ashbel and Seligman 1949, Seligman and Ashbel 1951). Lipoids with active carbonyl groups were stained in various endocrine tissues (Ashbel and Seligman 1949), in virilizing tumors of the human adrenal cortex (Seligman and Ashbel 1951a) and in interstitial and Sertoli cell tumors of the dog's testis (Ashbel, Cohen, and Seligman 1951). Evidence was presented for considering the carbonyl groups in the lipoid of these tumors to be ketonic rather than aldehydic and all the evidence in favor of the thesis that ketosteroids are responsible for the staining reaction has been summarized (Ashbel, Cohen and Seligman 1951). The reaction is by no means specific for ketosteroids, but only for active carbonyl groups which it has been shown by other reactions are ketonic. It is assumed that ketosteroids are responsible in major part for the staining reaction when it is present in tissues in which material possessing the solubility properties of the steroids is found and in which ketosteroids are known to be present.

The present communication deals with a study of the carbonyl reaction in fifteen non-virilizing tumors of the adrenal cortex of which six were associated with Cushing's syndrome.

Received for publication November 26, 1951.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

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METHOD

The method for demonstrating carbonyl groups in frozen sections of formalin-fixed tissue (Ashbel and Seligman 1949) was modified toward the end of this investigation in order to eliminate the diffuse violet or bluish stain, especially prominent in epithelial structures, which is due to the non-specific binding or adsorption of 2-hydroxy-3-naphthoic acid hydrazide to protein.[3]

This is done by washing the sections for ½ hour in ½ N hydrochloric acid after reaction with the hydrazide is complete, but before coupling with tetrazotized diorthoanisidine to form the blue dye is performed. Excess hydrazide is thus solubilized through the basic hydrazide group. A less complete removal of the stain is produced using in place of hydrochloric acid, 1% bicarbonate which solubilizes the hydrazide through the phenolic group whose acidity is enhanced by the ortho carbonyl group.[4] Neither the acid nor the bicarbonate wash altered the quality of the staining for carbonyl groups in myelin (Seligman and Ashbel 1951). In our original description of the method it was pointed out that the hydrazide reaction could be conducted at room temperature for 2 hours, or at 60° C. for 45 minutes. While the latter conditions effect an economy of time, superior sections are obtained if room temperatures for 2 hours are used. The following details for conducting the reaction are recommended: Reagents:[5]

1. 2-Hydroxy-3-naphthoic acid hydrazide (0.1%). The hydrazide (1 gram) is dissolved in 50 cc. of hot glacial acetic acid in a volumetric flask. To this is added 950 cc. of 50% alcohol by volume prepared just before use from absolute ethyl alcohol and distilled water. In order to prevent formation of aldehydes, unsealed bottles of absolute alcohol are stored in the refrigerator. The reagent may be stored at room temperature for 2 weeks.
2. Alcohol-buffer solution. Phosphate buffer (pH 7.2–7.5, 1/15 M) is mixed with an equal volume of absolute ethyl alcohol just before use.
3. Ethyl alcohol (50%).
4. Hydrochloric acid (0.5 N).
5. Sodium bicarbonate (1%).
6. Tetrazotized diorthoanisidine. The powder (50 mg.) is stirred into the alcohol buffer at the time of coupling without prior solution in water because the diazonium compound decomposes and darkens rapidly in pure aqueous solution.

Procedure:

1. Wash formalin from frozen sections in several changes of cool water for several hours. Sections may first be mounted on microscope slides and allowed to dry for 10 minutes.

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[3] The binding material was considered to be proteinaceous because the stain could not be removed by extraction with such organic solvents as acetone or xylene which readily extract ketosteroids. It was our impression that the intensity of this stain was more prominent in tissues fixed for a long time. This impression was confirmed in a recent report by Herman and Dempsey (1951), who studied the effect of long fixation in formalin on the reaction.

[4] Mrs. Edith Herman, Department of Anatomy, Harvard Medical School, was good enough to reexamine her material and, at our suggestion, in separate experiments, tried the hydrochloric acid and bicarbonate wash with the same results reported above.

[5] All reagents may be obtained from Dajac Laboratories, Monomer Polymer, Inc. 511 Lancaster Street, Leominster, Mass.

2. Incubate sections in the hydrazide solution at room temperature for 2 hours The maximum number of sections in 50 cc. of reagent is 10 to 20.
3. Wash in several changes of 50% alcohol for 2 hours.
4. Incubate in 0.5 N hydrochloric acid at room temperature for ½ hour optional.
5. Wash in water.
6. Rinse in 1% sodium bicarbonate.
7. Wash in water.
8. Place in alcohol-buffer solution. Add tetrazotized diorthoanisidine powder and stir. Stain for 2 minutes.
9. Wash in several changes of water.
10. Rinse in 50% alcohol followed by water in order to dislodge gas bubbles.
11. Mount in glycerogel.

The tumor material obtained from various hospitals, had been fixed in formalin from a few days to 17 years. Twelve of the fifteen tumors were diagnosed as carcinomas by the pathologists of the hospitals from which they were obtained. Six were known to be associated with Cushing's syndrome. The remainder were unsuspected before post-mortem examination or were discovered during surgical exploration for abdominal pain, or during splanchnicectomy. Sufficient data are lacking to determine whether there was associated virilization or Cushing's syndrome. Obvious signs and symptoms of such endocrinopathy were absent. The urinary ketosteroids had not been measured in most of the cases.

Numerous sections from several areas of each tumor were stained for lipoid with sudan IV and for ketosteroids by the foregoing methods. A particularly exhaustive search for ketosteroid was made when the usual search did not disclose their presence. Evidence for the lipoid nature and ketonic properties of the stainable material was obtained by the procedures described by us previously. Sections stained with sudan IV were photographed through a green filter and those stained for ketosteroids were photographed through a red filter.[6]

RESULTS

Adrenal Cortical Tumors with Cushing's Syndrome

Protocol data and the results of the reaction for carbonyl groups are given in order of decreasing period of fixation in Table 1. The symptomatology of six patients with adrenal tumors associated with Cushing's syndrome is given in Table 2. One tumor contained practically no lipoid (case 4), three contained spotty areas with a moderate number of lipoid droplets (cases 1, 2, and 3), and two contained abundant droplets of lipoid (cases 5 and 6). No reaction for carbonyl groups was observed in cases 1 and 4, a pale stippling within occasional cancer cells was observed in case 3 and only one area of the many inspected in the tumor of case 2 contained a few cells with blue stained droplets. A definite and consistent positive reaction for carbonyl groups was seen only in cases 5 and 6. These last two tumors were fixed in formalin for the shortest period of time. In case 5 the tumor cells had a lobular arrangement (figs. 1, 2, 3). Only a fraction of the tumor cells in each lobule contained fat (fig. 1)

[6] Photomicrographs by Mr. Leo Goodman.

TABLE 1. ADRENAL TUMORS WITH CUSHING'S SYNDROME

Case No.	Exp. No.	Hospital No.	Hospital	Sex	Age	Diagnosis	Period of fixation in formalin	24 Hour urinary output at time of operation		Weight of tumor of adrenal	Reaction for carbonyl groups in lipoid of tumor sections
								17-ketosteroid	Corticosteroids*		
					years		years	mg.	mg.	gms.	
1	282	A-32-208	Children's	F	10½	Pituitary basophilic adenoma and undifferentiated adrenal cortical carcinoma	17	—	—	700	negative
2	292	A-37-8090	Mass. General	F	36	Adrenal cortical carcinoma with metastases	12	20	—	—	positive in one area
	317	A-38-1725	Mass. General	F	56	Adrenal cortical carcinoma with lymph node metastases	11	74.0	—	1000	trace
4	267	45-82680	New Eng. Deaconess	M	25	Adrenal cortical adenoma	4	—	—	11	negative
5	289	49-13346	Mass. General	F	35	Adrenal cortical adenoma pituitary basophilism	0.3	2.3	0.49	16	positive
6	477	A-50-68	Beth Israel	F	50	Adrenal cortical carcinoma	0.05	20.5	1.5	600	positive

* Method of Talbot *et al.* (1947).

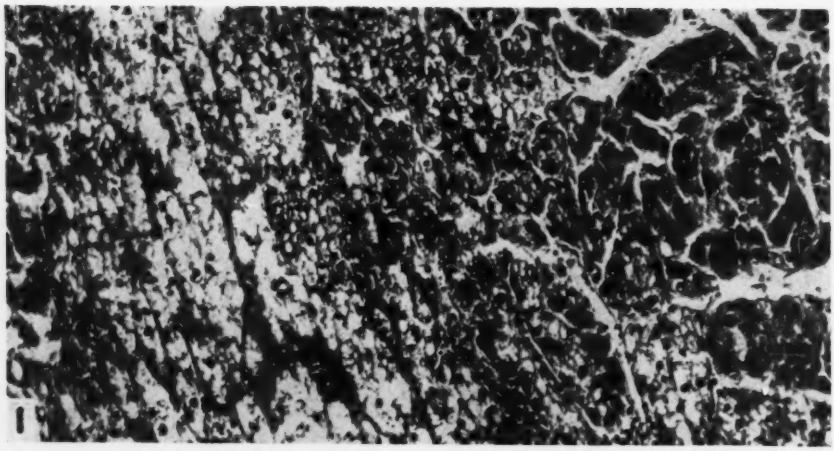
which reacted for carbonyl groups (fig. 2). In case 6 the tumor cells were undifferentiated (fig. 4), but contained large amounts of lipoid (fig. 5), which was positive for carbonyl groups (fig. 6). These positive tumors were subjected to the reactions for distinguishing ketonic from aldehydic material, as previously described for normal adrenal cortex and virilizing tumors (Seligman and Ashbel 1951, Seligman and Ashbel 1951a). As before, the stainable material proved to be ketonic and lipoid soluble and was, therefore, presumably ketosteroid.

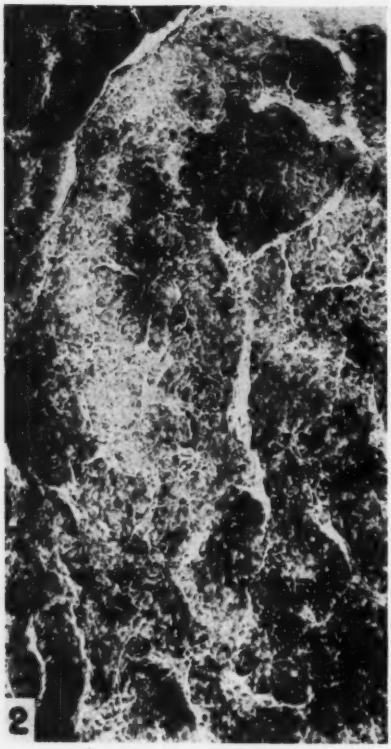
COMMENT

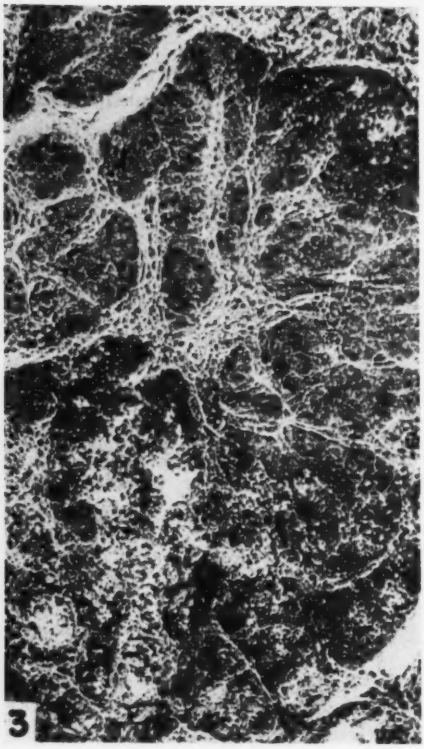
The two tumors (cases 6 and 5) which contained large amounts of

TABLE 2. FINDINGS IN SIX PATIENTS WITH CUSHING'S SYNDROME

Case no.	
1	Headache, abdominal pain, hypertension, diabetes, cardio-renal disease, moonshaped face, excessive hair around genitalia.
2	Abdominal pain, hirsutism, headache, hypertension, edema, moonshaped face, husky voice, telangiectasia, diabetes, osteoporosis, abdominal striae.
3	Pain in lower thoracic spine, dyspnea, palpitations, hirsutism of face, edema, hypertension.
4	Back pain, craving for sweets, weight increase of 40 lbs., emotional depression, irritability, ease of exhaustion, and sleepiness, moonshaped face, striae, abnormal glucose tolerance, decrease of muscle mass of extremities, kyphosis normal blood pressure.
5	Right upper quadrant abdominal pain, weakness of legs and atrophy of muscles, headaches, hypertension, dizziness, husky voice, full face with capillary telangiectasia, ecchymoses over arms, legs, shoulders, and breasts, obesity of trunk, distended neck veins.
6	Hypertension, headaches, osteoporosis, puffy face, hirsutism, edema, purple striae over trunk, trunk obesity, ecchymosis over hips and lower extremities, thickening of subcutaneous tissue over upper dorsal and lower cervical vertebrae (buffalo hump), diabetes.







ketosteroid had been fixed in formalin for 2.5 and 15 weeks. The other four tumors had been fixed in formalin for 4–17 years. The weak to absent staining in these may have been due to the significant water solubility of the 17-hydroxy-corticosteroids, which produce Cushing's syndrome. The water soluble property of the 17-hydroxy-corticosteroids has been used in urine assay for their separation from the 17-ketosteroids by partition between water and benzene (Venning, Hoffman and Browne 1944, Talbot, Albright, Saltzman *et al.*, 1847). Partition between water and lipoid in tissues containing steroids dissolved in neutral fat, phosphatides, cholesterol and cholesterol esters is not likely to be as efficient as partition between benzene and water. But, in the course of years in formalin, depletion of the slightly water soluble steroids could have occurred. In cases 5 and 6 the period of fixation was too short for significant extraction of the hydroxy corticosteroids. The positive test for ketosteroid in a few sections of cases 2 and 3 was probably due to the 17-ketosteroids (androgens) and progesterone which would not be expected to move from the lipoid droplets to water. This view is supported by the study of adrenal tumors associated with virilism and precocious development. The androgenic ketosteroids of these tumors were readily demonstrated in tissue fixed for as long as 11 years (Seligman and Ashbel 1951a).

OTHER ADRENAL TUMORS

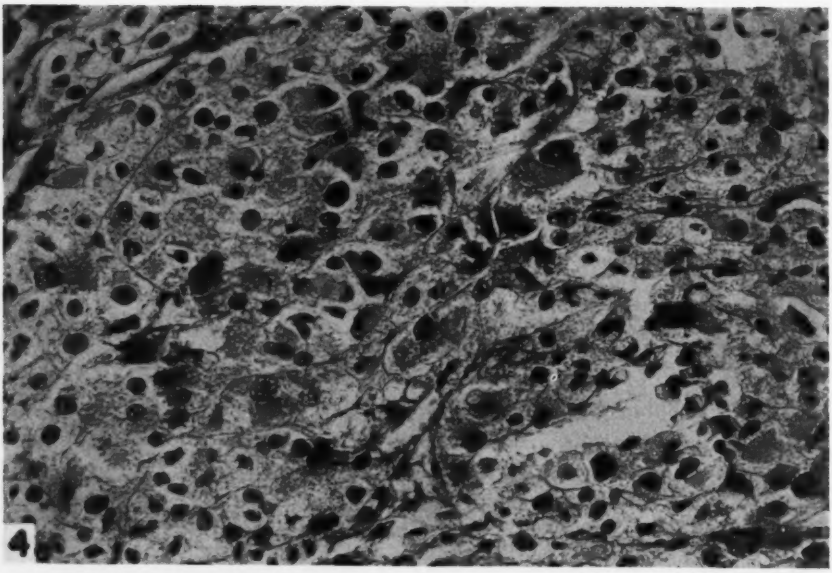
Protocol data and the results of the test for carbonyl groups are given in Table 3. Three (Nos. 8, 9, 12) of the nine tumors did not react for carbonyl groups. One was an embryonal carcinoma of the adrenal cortex in a 2 year old child, and contained sudanophilic droplets. These tumors had been fixed in formalin for 11, 7, and 4 years respectively. Three other tumors (Nos. 7, 10, 11) which had been fixed in formalin for 12, 6 and 5 years respectively did react for carbonyl groups. Sudanophilic material was present in all tumors except No. 9.

Tumor 7 (Figs. 7–10) was an adrenal cortical carcinoma with metastases to lung and liver in an elderly male who died soon after admission. Evidence of virilism was not noted, but this does not exclude androgenic or estrogenic ketosteroids in the tumor. Carbonyl groups were readily demonstrated in the lipoid of the metastases as well as of the primary

FIG. 1. Adrenal cortical adenoma associated with Cushing's syndrome (case 5). The tumor cells contain variable amounts of lipoid. Lipoid laden cells at left, moderate (center), none (upper right). Hematoxylin-eosin. ×250.

FIG. 2. Same as fig. 1. Sudan IV. ×250.

FIG. 3. Same as figs. 1 and 2. Reaction for carbonyl groups. Fixed in formalin for 15 weeks. ×250.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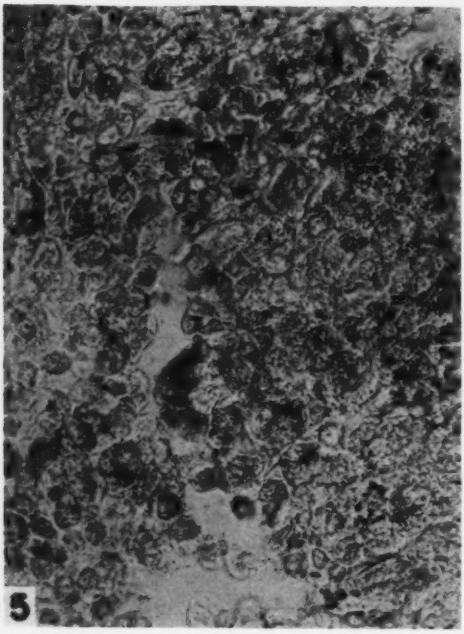
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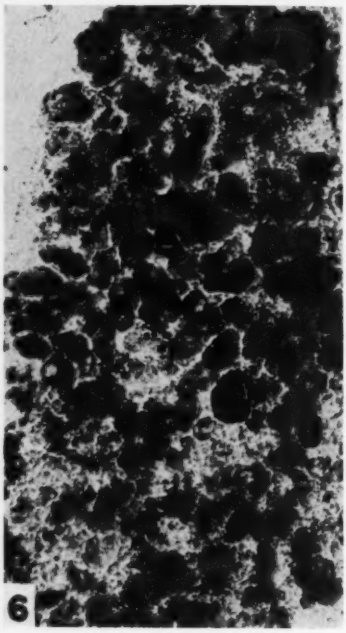
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tumor in spite of 12 years of fixation in formalin. Corticosteroids were therefore probably not responsible for the carbonyl reaction.

Tumor 10 was a small adrenal cortical carcinoma discovered at a laparotomy for abdominal pain in a 30 year old woman. Since the reaction for carbonyl groups in lipoid was intense after fixation in formalin for 6 years, it must have been due to other ketosteroids than the more water soluble corticosteroids.

Tumor 11 was a small adrenal cortical carcinoma in an elderly female who was admitted to the hospital in coma. She died of cerebral infarction. This tumor was fixed in formalin for 6 years and contained lipoid which reacted for carbonyl groups (figs. 11–13).

Three adrenal cortical carcinomas (Nos. 13, 14, 15) were discovered in adults at exploration of the splanchnic nerve, at autopsy, and at laparotomy for abdominal pain respectively and had been fixed for 3 years, 0.5 year, and 1 day respectively. They contained lipoid and reacted for carbonyl groups.

TABLE 3. ADRENAL TUMORS *not* ASSOCIATED WITH VIRILISM OR CUSHING'S SYNDROME

Case No.	Exp. No.	Hospital No.	Hospital	Sex	Age	Diagnosis	Period of fixation in formalin	Reaction for fat in tumor section	Weight of tumor of adrenal	Reaction for carbonyl groups in lipoid of tumor sections
					years		years		gms.	
7	202	A-37-697	Boston City	M	55–60	Adrenal cortical carcinoma with metastases	12	positive	2000	positive with positive metastases
10	269	22466	New Eng. Deaconess	F	30	Adrenal cortical carcinoma	6	positive	14	positive
11	203	A-43-484	Boston City	F	63	Adrenal cortical carcinoma	6	positive	6	positive
8	264	A38997	New Eng. Deaconess	F	56	Adrenal cortical adenoma	11	positive	50	negative
9	283	A-42-190	Children's	M	2	Embryonal carcinoma of adrenal cortex involving viscera	7	sparse	2000	negative
12	199	A-45-49	Boston City	F	72	Adenocarcinoma of adrenal with metastases to skull and spine	4	positive	7	negative
13	270	S91115	Lahey Clinic	F	41	Adrenal cortical carcinoma	3	positive	3.5	positive
14	321	A-51-49	New Eng. Deaconess	M	66	Adrenal cortical carcinoma with metastases	0.5	positive	2	positive
15	297	116752	New Eng. Deaconess	M	56	Adrenal cortical carcinoma	1/365	positive	1500	positive

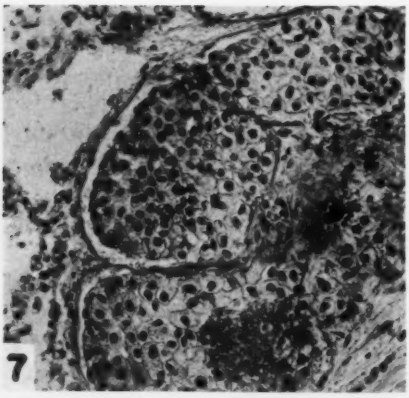
COMMENT

On the basis of the results with adrenal tumors known to be associated with either Cushing's syndrome or virilization (Seligman and Ashbel 1951a), it can be concluded that in tumors 8, 9, and 12 which were fixed

FIG. 4. Adrenal cortical carcinoma associated with Cushing's syndrome (case 6). The tumor cells are pleomorphic and contain variable amounts of lipoid. Hematoxylin-eosin. ×400.

FIG. 5. Same as fig. 4. Area with little lipoid. Sudan IV. ×400.

FIG. 6. Same as figs. 4 and 5. Reaction for carbonyl groups. Fixed in formalin for 2.5 weeks. ×400.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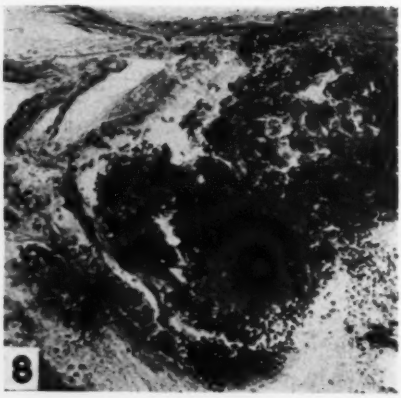
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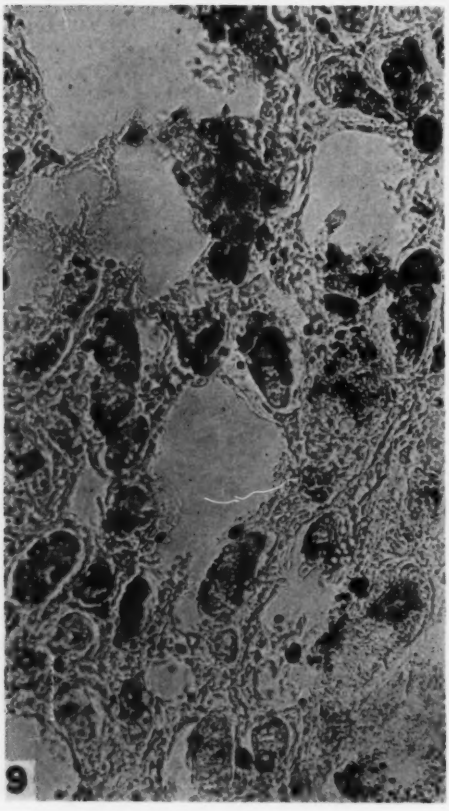
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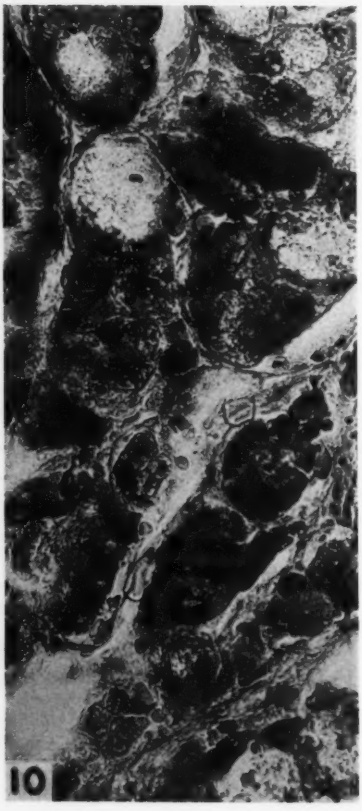
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for 4 years or longer and did not disclose ketonic carbonyl groups, androgenic ketosteroids were never present. Corticosteroids might have been produced or the tumors may not have produced either class of hormones. The three tumors (Nos. 7, 10, 11) fixed for 6 years or longer which reacted for carbonyl groups must have contained ketosteroids other than the corticosteroids, such as the androgens or progesterone. The three positive tumors (Nos. 13, 14, 15) which were fixed for shorter periods of time might have contained any of the ketosteroids. The histochemical findings in these nine tumors cannot be correlated with the presence or absence of virilism or Cushing's syndrome because pertinent data in most cases were not obtainable.

DISCUSSION

It thus appears that adrenal cortical tumors associated with Cushing's syndrome, and which elaborate large amounts of the corticosteroids (compounds E and F), may show no ketonic carbonyl groups (ketosteroids) when the tissue is fixed in formalin for four years or longer. On the other hand, adrenal cortical tumors associated with precocious development and virilism give positive reactions for ketonic carbonyl groups (ketosteroid) even after fixation in formalin for as long as eleven years (Seligman and Ashbel 1951). The ketosteroids most likely responsible for the virilization are the highly lipoid-soluble androgens. These ketosteroids as well as progesterone would not be extracted from the lipoid droplets in which they are dissolved by a long period of incubation in aqueous medium, whereas the corticosteroids, particularly the 17-hydroxy and 11-hydroxy derivatives, have a significant solubility in water.

In a histochemical study of interstitial and sertoli cell tumors of the testicle of the dog, incubation of frozen sections for 24 hours at pH 11 removed the carbonyl containing lipoid from the sertoli cell tumors (estrone) but not from the interstitial cell tumors (androgen) (Ashbel, Cohen and Seligman 1951). This is consistent with the phenolic character of the ketosteroid, estrone, and correlates with the reported feminizing properties of Sertoli cell tumors in dogs.

The close correlation of the results of histochemical analysis with what

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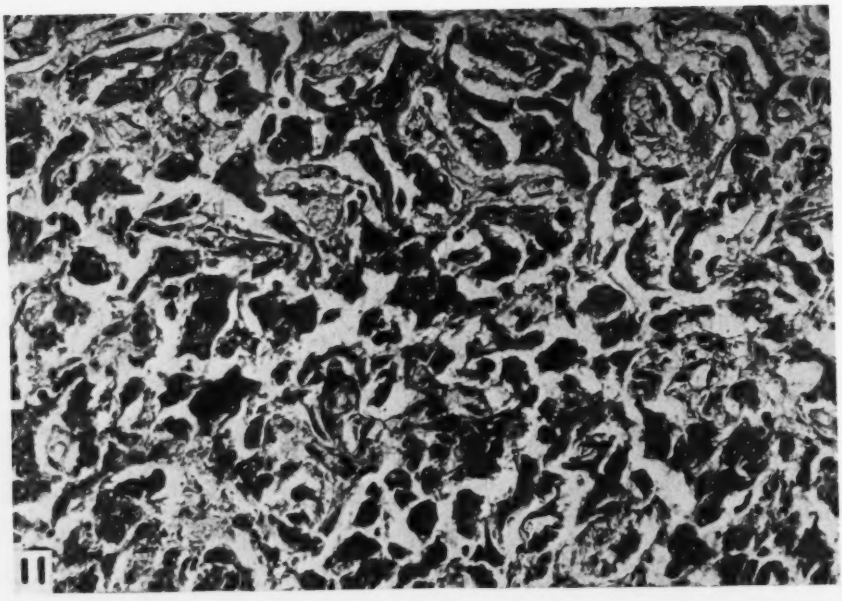
FIG. 7. Adrenal cortical carcinoma metastatic to lung without Cushing's syndrome (case 7). Hematoxylin-eosin. ×240.

FIG. 8. Same as Fig. 7. Reaction for carbonyl groups. Fixed in formalin for 12 years. ×240.

FIG. 9. Adrenal cortical carcinoma metastatic to liver without Cushing's syndrome (case 7). Tumor cells containing lipoid. Sudan IV. ×400.

FIG. 10. Same as fig. 9. Reaction for carbonyl groups. Fixed in formalin for 12 years. ×400.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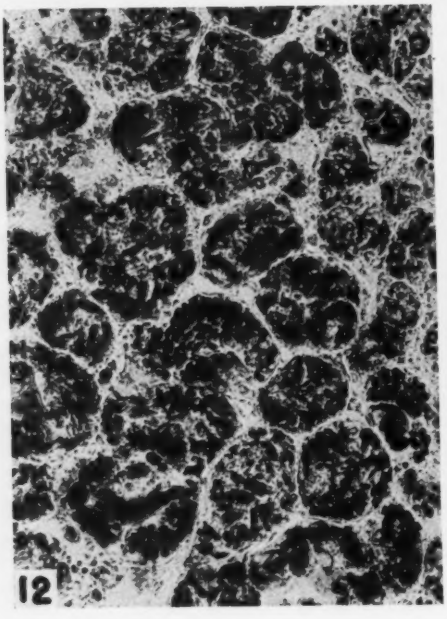
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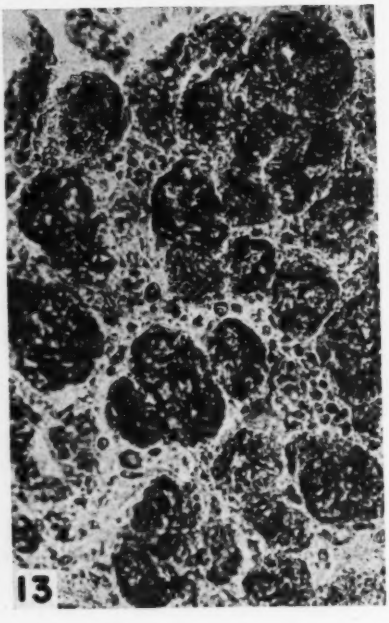
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is known of the properties of the ketosteroids further confirms our view that ketonic lipoidal material demonstrated in these tissues are in fact ketosteroids.

SUMMARY

Adrenal cortical tumors associated with Cushing's syndrome contained active lipoidal ketonic carbonyl groups (presumably corticosteroids) when fixed in formalin for brief periods (months) and little or no carbonyl groups (presumably other ketosteroids) when fixed in formalin for years. The solubility of the corticosteroids in water though slight is believed to explain this phenomenon.

Six of nine adrenal cortical tumors not known to be associated with Cushing's tumor or virilism contained active ketonic carbonyl groups in the lipoid droplets of the tumor cells (presumably one or more of the ketosteroids).

Acknowledgement

Acknowledgment for the tumors, their identification and the clinical data is due Drs. Monroe Schlesinger and Leopold Reiner, Beth Israel Hospital; Dr. Sidney Farber, Children's Hospital, Boston; Dr. Benjamin Castleman, Mass. General Hospital; Drs. Shields Warren and Samuel Hicks, New England Deaconess Hospital, Boston, and Dr. Stanley Robbins, Mallory Institute, Boston City Hospital.

Acknowledgment for technical assistance is due Marie Mollomo Dobson, Nancy Appel, Irving Siegel and Myron Milden.

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FIG. 11. Adrenal cortical carcinoma without Cushing's syndrome (case 11). Tumor cells are pleomorphic. Hematoxylin-eosin. ×400.
FIG. 12. Same as fig. 11. Sudan IV. ×400.
FIG. 13. Same as figs. 11 and 12. Reaction for carbonyl groups. Fixed in formalin for 6 years. ×400.

ANTIDIURETIC HORMONE IN THE SERUM OR PLASMA OF RATS[1]

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THE recent reports of Birnie and his colleagues were the first describing the antidiuretic action of serum of normal or adrenalectomized rats (Birnie *et al.*, 1949 and 1950). Their results suggested that antidiuretic hormone of neurohypophysial origin can be detected in rather high concentration in rat serum especially after adrenalectomy. Subsequently, Dicker and Ginsburg (1950) concluded that heparinized plasma of rats lacks the antidiuretic substance which can be detected in serum. Their report implied that the antidiuretic substance is an artifact of the clotting process.

It seemed important to reinvestigate various aspects of these findings. Does circulating blood in the normal rat contain antidiuretic hormone in relatively enormous concentrations represented by approximately 0.75 milli-unit of Pitressin per ml. of serum?[2] Can a more reliable estimate of the amount of antidiuretic substance in serum be made than is possible if assays are performed by intraperitoneal or subcutaneous injection into rats? Is the concentration markedly increased following adrenalectomy? Is the antidiuretic substance absent from heparinized plasma? Does the serum of kangaroo rats (*Dipodomys merriami*) which may excrete comparatively high concentrations of antidiuretic hormone in the urine (Ames and van Dyke, 1950) contain appreciably higher concentrations of antidiuretic substance than the serum of laboratory rats? Efforts to answer these principal questions are described in this report.

METHODS

Assays of antidiuretic activity in specimens of serum or plasma were carried out in male Long-Evans rats by a modification of the intravenous method described by Jeffers, Livezey and Austin, (1942). The assay animals were rendered diuretic by the admin-

Received for publication November 26, 1951.

[1] This investigation was in part supported by grants from the Playtex Park Research Institute and E. R. Squibb and Sons.

[2] This figure was reported by Dicker and Ginsburg. In neither of their publications did Birnie *et al.* report assays in terms of a standard; however, their results are not in serious disagreement with the estimate of Dicker and Ginsburg.

istration of water and ethanol which anesthetized the rats and probably suppressed the secretion of endogenous antidiuretic hormone (van Dyke and Ames 1951). After the urethra had been ligated a shortened ureteral catheter (3 mm. O.D.) was inserted through a cystotomy and firmly anchored by a ligature. Urine was then collected at 5 to 10 minute intervals directly from the bladder into specially-designed long 5 ml. cylinders graduated to 0.05 ml. All injections were made intravenously through a 24 gauge needle which was inserted a distance of 20 mm. into the saphenous vein. The needle contained a wire mandrel which could be removed before attaching a syringe. Both the needle and the leg were firmly held by adhesive tape. Estimations of antidiuretic activity were made by comparing the antidiuretic effect of the materials to be assayed with that obtained from known amounts of Pitressin in the same assay rat. Fig. 1 demonstrates a typical curve. In all acceptable assays, 0.020 m.u. of Pitressin could be detected. (Occasionally, the routine oral dose of water, 30 minutes after aqueous ethanol, was partly aspirated owing to depression of normal reflexes by the ethanol; there was no diuresis in such animals which had to be discarded.) The precision of this assay method was such that in

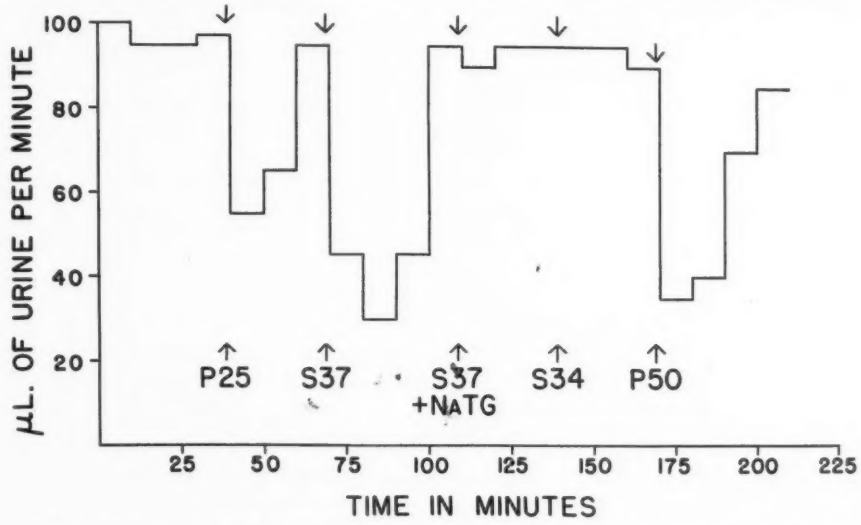

FIG. 1. An example of the assay of antidiuretic hormone by the intravenous method in the rat. At −95 minutes, 5 ml. of 9.6 (v/v) per cent of ethanol per 100 gms. were administered by stomach tube; at −65 minutes 3 ml. of water per 100 gms. were similarly administered.

P25 and P50: 25 and 50 μu. of Pitressin.

S37: equivalent of 0.1 ml. of serum of a kangaroo rat anesthetized by ether and then decapitated.

S37+NaTG: equivalent of 0.3 ml. of serum 37 containing 0.01 M sodium thioglycollate.

S34: 0.5 ml. of serum of a kangaroo rat decapitated without ether or exceitment.

No antidiuretic hormone could be detected in serum after simple decapitation (S34); about 0.5 m.u. per ml. of serum was present after ether anesthesia (S37) and was inactivated by thioglycollate.

the usual dose-range of 0.020 to 0.1 m.u., increments or decrements of 0.020 m.u. could be distinguished. Duplicate assays of the same dose in the same animal were in excellent agreement.

The method of Birnie *et al.* (1950) was also used for the assay of several specimens of serum. In this method, rats are hydrated by three successive doses of water and the serum or diluted Pitressin is injected intraperitoneally.

Blood was secured by four methods: heart puncture without anesthesia, decapitation without anesthesia, and heart puncture or decapitation under ether anesthesia Struggling always occurred with the first method; it also was uniformly present before the induction of surgical anesthesia by ether after which heart puncture or decapitation was performed. In the second method, simple decapitation, the animals were handled gently for 5 to 10 minutes and when they appeared tranquil the necks were swiftly severed by large, sharp surgical scissors; blood was collected from the trunks suspended over small funnels in centrifuge tubes. Clotting was prevented in some of the samples of blood by adding to the collecting tubes a minute volume of a concentrated solution of sodium heparin so that 10 units of heparin per ml. of blood were present. All blood specimens were centrifuged for 2 minutes and the serum or heparinized plasma was removed immediately. Injections for assay were made within 10 minutes after the blood was obtained unless a longer period was deliberately chosen.

Bilateral adrenalectomy was performed as described by Ingle and Griffith (1949). The adrenalectomized animals were maintained on 1 per cent NaCl before they were used in experiments. Except where indicated, the saline solution was replaced by water 4 or 5 days prior to the rats' use in experiments. After each experiment with adrenalectomized rats the suprarenal and perirenal tissue was examined grossly and microscopically. Experiments performed with rats in which suspicious tissue was present were discarded.

RESULTS

The results of various experiments with Long-Evans rats are summarized in Table 1. Sera from 14 normal adult rats were assayed for antidiuretic activity by the intravenous method. The rats had been bred in this laboratory and handled frequently; they were neither anxious nor excited before blood was obtained by sudden decapitation. Six of the specimens were tested by both the intravenous and intraperitoneal method of assay. In no case was antidiuretic activity detected under these conditions by either method of assay (group A). On the other hand, when blood was obtained by heart puncture from 14 similar animals which struggled and became excited, the sera contained antidiuretic activity which was equivalent to from 0.10 to 0.50 m.u. of Pitressin per ml. with a mean of 0.17 ± 0.029 m.u./ml. (group C). Three additional rats were hydrated with 5 ml. of water per 100 gm. of body weight one hour before heart puncture. Sera from these rats had antidiuretic activity in the same range as rats without previous hydration.

The effect of ether anesthesia prior to obtaining blood by heart puncture or decapitation was also observed (group E). Four rats were decapitated and 5 had cardiac punctures under ether anesthesia. The antidiuretic

TABLE 1. THE CONCENTRATION OF ANTIDIURETIC ACTIVITY IN THE SERUM OF LONG-EVANS RATS. (THE INTRAVENOUS METHOD WAS USED FOR ALL THE ASSAYS.)

Method	Normal rats			Adrenalectomized rats		
	Group	No. of rats	Mean ±S. E.[1] m.u./ml. serum	Group	No. of rats	Mean ±S. E.[1] m.u./ml. serum
Decapitation without anesthesia	A	14	0[2]	B	14	0.007[3]
Heart puncture without anesthesia	C	14	0.17 ±0.029	D	22	0.25 ±0.020
Decapitation or heart puncture under ether anesthesia	E	9	0.29 ±0.042	F	8	0.21 ±0.006
Heart puncture after ethanol	G	13	0.02 ±0.015	H	5	0.08 ±0.037
	Hypophysectomized rats					
Heart puncture without anesthesia	I-1	11	0[4]			
Decapitation under ether anesthesia	I-2	12	0[4]			

[1] S. E. = Standard error of mean.
[2] Assay of 0.5 ml. of serum from 10 rats (<0.04 m.u./ml.) and of 1.0 ml. of serum from 4 rats (<0.02 m.u./ml.).
[3] Serum 1 rat contained 0.1 m.u./ml.; all the others contained no detectable activity (usually 0.5 ml. of serum assayed).
[4] Assay of 1.0 ml. of serum in all rats (<0.02 m.u./ml.).

activity of these sera ranged from 0.10 to 0.40 m.u./ml. with a mean of 0.29 ±0.042 m.u./ml. The two methods of obtaining blood did not differ in their effect on the antidiuretic titer of serum following ether anesthesia. In some experiments, heart puncture was immediately followed by decapitation; the concentration of antidiuretic activity was identical in these successive samples of serum from a given animal. Ether anesthesia did not modify the results.

Since ethanol probably suppresses the secretion of antidiuretic hormone (see van Dyke and Ames, 1951) the antidiuretic activity of sera from 13 rats under ethanol anesthesia was determined. One hour or more before heart puncture 5 ml. of 9.6 (v/v) per cent ethanol in water per 100 gm. body weight were given by stomach tube. There was no period of excitation except in response to gavage. The antidiuretic activity was significantly less than in the sera obtained by heart puncture from unanesthetized rats or those subjected to ether anesthesia. The titer of 9 specimens was less than 0.02 m.u./ml. and the mean was 0.02 ±0.015 m.u./ml. or less than one tenth of that found under ether anesthesia (group G). Just before blood samples were obtained from 3 additional rats, they were placed in an ether chamber with control rats until the latter were anesthetized. Assays of their sera were higher than the majority of sera from rats which received ethanol alone.

Dicker and Ginsburg (1950) have reported the presence of an antidiuretic substance in the serum but not in the plasma of rats under ether anesthesia. Portions of 3 of the blood samples obtained by heart puncture were collected in tubes containing heparin (10 u./ml.). The antidiuretic activity of these plasma samples did not differ from samples of sera collected from the same animal at the same time. The mixing of sera from 4 rats with heparin did not change its antidiuretic activity and 15 u. of heparin alone did not affect the diuresis of 3 assay rats. Dicker and Ginsburg mentioned that unpublished experiments of Ginsburg and Heller revealed that the antidiuretic action of rat serum could not be attributed to the serotonin of Rapport. Serotonin was kindly furnished to us by Dr. Rapport; doses of 4 or 6 μgm. intravenously had no antidiuretic effect in rats.

In an attempt to identify the source of the antidiuretic substance, sera from 23 adult hypophysectomized rats, 3 to 4 weeks after operation, were assayed. In 11 animals blood was obtained by heart puncture without anesthesia (group I-1); the remaining 12 animals were anesthetized with ether and then decapitated (group I-2). In neither group could any antidiuretic activity be detected in 1 ml. samples of serum. The assay-rats all responded to 0.020 m.u. of Pitressin; therefore, the serum of the hypophysectomized rats contained less than this quantity of antidiuretic principle per ml. These results confirm the conclusion of Birnie *et al.* (1950).

The antidiuretic activity of rat serum has been reported to be higher than normal in adrenalectomized animals (Birnie *et al.*, 1949, 1950). The experiments to be reported in this section were performed in adrenalectomized rats about 1 to 2 weeks after operation; neither sodium-chloride solution nor any other treatment was available to these animals for at least 4 days before experiments. The 14 animals of group B, Table 1, were decapitated in a tranquil state without anesthesia and in only one serum sample was it possible to demonstrate antidiuretic activity (0.1 m.u. per ml.). Serum obtained by cardiac puncture from 22 adrenalectomized rats had activity equivalent to from 0.10 to 0.50 m.u. of Pitressin per ml. with an average of 0.25 ± 0.02 m.u./ml. following heart puncture (group D). All but 3 of these rats did not receive 1 per cent NaCl for 5 or more days and the 3 specimens from rats on saline did not differ from the others. This was also true of sera from 2 rats which received 5 ml. of water per 100 gm. of body weight one hour before heart puncture. The antidiuretic titer of serum obtained by decapitation immediately following heart puncture did not differ from that of the corresponding cardiac blood of the same animal. Ether anesthesia was induced in 8 adrenalectomized

TABLE 2. THE CONCENTRATION OF ANTIDIURETIC ACTIVITY IN THE SERUM OF KANGAROO RATS (*Dipodomys merriami*). (THE INTRAVENOUS METHOD WAS USED FOR ALL THE ASSAYS.)

Kangaroo rats			
Method	Group	No. of rats	Mean ± S. E.[1] m.u./ml. serum
Decapitation without anesthesia	J	20	0.06[2] ± 0.010
Heart puncture without anesthesia	K	4	1.25 ± 0.34
Decapitation or heart puncture under ether anesthesia	L	16	0.75 ± 0.120

[1] S. E. = Standard error of mean.
[2] Sera from 8 rats contained less than 0.040 m.u./ml.

rats before heart puncture or decapitation (group F). The antidiuretic activity ranged between 0.19 and 0.23 m.u./ml. with a mean of 0.21 ± 0.006 m.u./ml. The effect of the ethanol anesthesia in the adrenalectomized rats was similar to that of the intact rats. The antidiuretic activity ranged from 0 to 0.20 m.u./ml. with a mean of 0.08 ± 0.037 m.u./ml.

The kangaroo rat, *Dipodomys merriami*, was studied in a manner similar to the normal rats (Table 2). These desert rodents, which had been captured recently, ordinarily obtain little or no water. In previous experiments it had been shown that kangaroo rat urine contains comparatively large amounts of antidiuretic activity (Ames and van Dyke, 1950). Twenty were decapitated in as tranquil a state as possible but these animals were apprehensive and were not as accustomed to laboratory conditions as the Long-Evans strain (group J). Eight sera had no detectable antidiuretic activity; the highest level was 0.19 m.u./ml. and the average value was 0.06 ± 0.010 m.u./ml. When heart puncture without anesthesia was performed in 4 animals the sera contained much more antidiuretic activity with a range of 0.75 to 2.25 m.u./ml. and a mean of 1.25 ± 0.34 m.u./ml. (group K). Serum from blood obtained under ether anesthesia by cardiac puncture or after decapitation also had marked antidiuretic activity. In these sera the range was equivalent to 0.38 to 2.0 m.u. of Pitressin per ml. and the mean was 0.75 ± 0.120 m.u./ml. Samples of serum from the same kangaroo rat collected by decapitation immediately after heart puncture contained the same concentration of antidiuretic activity.

Pitressin and the antidiuretic substance in the urine of dogs whose neurohypophysis was stimulated could be inactivated by freshly neutralized thioglycollic acid (Ames, Moore and van Dyke 1950). Samples of active sera from normal or adrenalectomized rats were mixed with a solution of sodium thioglycollate (1 M) to a final concentration of 0.01 M, and the mixture was allowed to stand at room temperature for 10 to 20 minutes. In every case the antidiuretic activity was completely lost. Speci-

mens of kangaroo rat serum also completely lost their activity when mixed with sodium thioglycollate. When sera without thioglycollate were allowed to stand at room temperature as much as 50 per cent of the activity was lost in 10 minutes; losses of activity were 50 to 75 per cent after 20 minutes and 50 to 100 per cent after 60 minutes.

Birnie *et al.* (1950) described their exact technic of assaying antidiuretic principle in rats. For purposes of comparison this time-consuming method was followed in 6 complete experiments with Long-Evans rats. In 5 of these experiments, the intravenous and intraperitoneal methods were compared. The results of one experiment are illustrated in Figure 2. In our hands, the intraperitoneal method is insensitive as illustrated by our inability to distinguish 1 m.u. of Pitressin from twice this dose in another assay-rat. In neither the intravenous nor the intraperitoneal method was there evidence of altered antidiuretic response to Pitressin mixed with inactive serum. Samples of sera from 12 adrenalectomized rats and from 6 normal rats decapitated in a tranquil state were found to contain no antidiuretic principle by either intraperitoneal or intra-

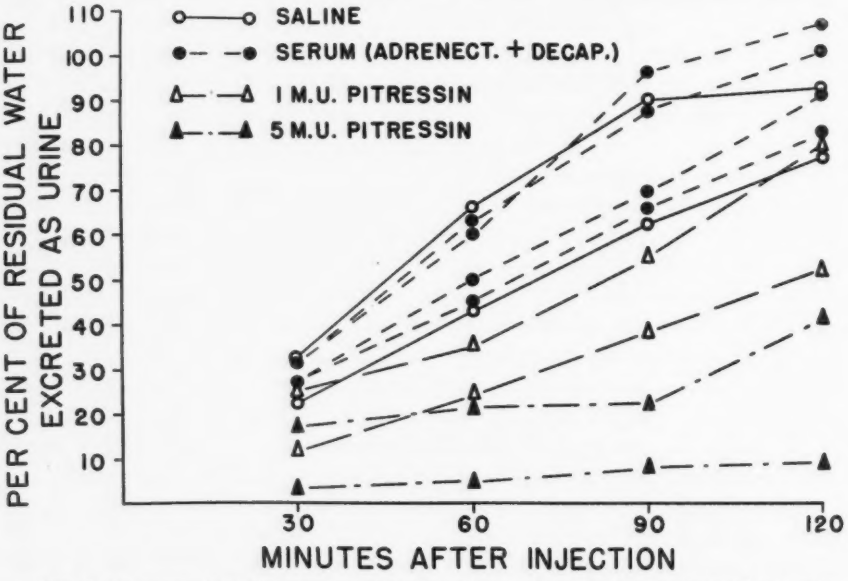

FIG. 2. An example of the results in individual rats after the intraperitoneal injection of serum or Pitressin by the assay-method of Birnie *et al.* (1950). In all such experiments, some control rats received saline and others Pitressin; the volume of all intraperitoneal doses was 1 ml. The serum of adrenalectomized rats decapitated in a tranquil state had no antidiuretic effect and this result agreed with intravenous assays of the same sera. However, the intraperitoneal method is not sensitive.

venous assay. More serious is the lack of correlation between the intravenous and intraperitoneal assays when sera containing endogenously liberated antidiuretic principle are compared. For example, 2 sera from adrenalectomized rats decapitated under ether anesthesia contained the equivalent of 0.20 m.u. of Pitressin per ml. by intravenous assay whereas by intraperitoneal assay the antidiuretic effect of 1 ml. of both sera was slightly less than that of 5 m.u. of Pitressin.

DISCUSSION

For the determination of antidiuretic hormone in biological fluids, the authors believe that an intravenous method is the best. In the experiments reported here reliance has been placed on an intravenous assay in rats, a method which is sensitive, probably specific, fairly accurate and not extremely time-consuming. By the intravenous method control observations with a standard preparation can be made in the same animal receiving material of unknown potency. On the other hand the method used by Birnie *et al.* (1950), is, in our experience, disadvantageous in respect of all five of the qualities mentioned for the intravenous assay. The well-known method of assay recommended by Burn is reliable if posterior-lobe *extracts* are used. However, it is not suitable for samples of rat serum such as were used in the work reported here: Burn's method requires 16 rats on each dose; the sensitivity is too low by subcutaneous injection except that it may be modified by impaired absorption if protein is also present; there is a possibility of a non-specific effect on diuresis.

Of fundamental importance is the source of the antidiuretic substance *when present in serum*. It was not possible to confirm the conclusion of Dicker and Ginsburg (1950) that heparinization of plasma prevents the detection of antidiuretic activity in plasma. The antidiuretic activity of serum or heparinized plasma from the same rat is identical. No antidiuretic activity is found in the serum of hypophysectomized rats (Groups I-1 and I-2, Table 1; confirming Birnie *et al.*, 1949 and 1950). Antidiuretic activity in plasma or serum is inactivated in the presence of 0.01 M thioglycollate. It is concluded that the antidiuretic substance is probably secreted by the neurohypophysis.

From the reports of Birnie and his colleagues, it was difficult to understand how the homeostatic control of water metabolism in the rat was possible if, as they contended, large amounts of antidiuretic principle were in the circulating blood. The explanation became evident when blood was obtained from the trunk after the rapid decapitation of tranquil rats. Serum from such blood contained no detectable antidiuretic activity whether obtained from Long-Evans rats which were normal (14

of 14) or had been adrenalectomized (13 of 14). Kangaroo rats, which can excrete high concentrations of antidiuretic hormone in the urine, reflecting a high potential rate of secretion into the blood, were similarly treated. The sera of 8 animals contained no detectable antidiuretic activity; however, appreciable amounts were present in the sera of the remaining 12 animals (0.05–0.20 m.u. per ml.). These animals had been captured only a few weeks previously. Despite gentle handling, they appeared ill at ease and it is believed that the secretion of antidiuretic hormone was thus facilitated in 12 of the 20 animals.

Comparisons of various experimental groups are summarized in Table 3. The P values listed for each comparison indicate the probability that

TABLE 3. COMPARISON OF MEANS OF GROUPS OF TABLES 1 AND 2

Groups compared	P*	Groups compared	P*
C and D	0.03	D and H	0.001
E and F	0.3	F and H	<0.01
G and H	0.1	C and K	<0.001
C and E	0.03	E and L	0.01
C and G	<0.001	J and K	<0.001
E and G	<0.001	J and L	<0.001
D and F	>0.1		

* Calculated according to Snedecor (1950).

the two samples are derived from the same population. P is 0.1 or greater in three of the comparisons and it is therefore probable that the two groups compared in each instance are similar. In all the other comparisons, it is unlikely (P = 0.001 or less to 0.03) that as great a difference in mean values would be encountered if two random samples from the same population were examined.

Ether anesthesia was used in gathering blood in all the experiments of Birnie *et al.* and Dicker and Ginsburg. (As will be pointed out, heart puncture without ether anesthesia is not an adequate control of the effect of the anesthetic as was believed by Birnie *et al.*) Decapitation is preceded by the secretion of antidiuretic principle into the blood if the "operation" is performed under ether anesthesia (Table 1: 0.2 to 0.5 m.u. per ml. serum in 5 normal rats and 0.25 to 0.5 m.u. per ml. serum in 4 adrenalectomized rats). The serum of blood obtained by heart puncture under ether anesthesia in the remaining animals of groups E and F, as would be expected, always contained antidiuretic hormone. Also without exception, the serum of heart-puncture blood collected without anesthesia (Table 1, groups C and D) always contained antidiuretic hormone (0.1 to 0.5 m.u. per ml. serum). Therefore, the belief of Birnie *et al.* (1950) that ether anesthesia does not affect the titer of serum antidiuretic

hormone is not valid. Both Rydin and Verney (1938) and O'Connor and Verney (1942) reported that painful or unpleasant stimuli appeared to be associated with the secretion of antidiuretic hormone in the dog. Recent human experiments by Kelsall (1949) led to a similar conclusion. It is our belief that heart puncture or ether anesthesia preceded by a stage of pain or excitement can lead to the secretion of considerable quantities of antidiuretic hormone because of central excitation of the neurohypophysis. The hormone cannot be detected in the serum of normal rats and only rarely in the serum of untreated adrenalectomized rats if there has been no preceding pain or excitement.

The experiments with kangaroo rats (Table 2) call for additional comment. High concentrations of antidiuretic hormone were detected in the serum after ether anesthesia or heart puncture or both (0.4 to 2.25 m.u. per ml. serum in 19 kangaroo rats and 0.15 m.u. per ml. in 1). It seems safe to conclude that these animals, like the Long-Evans rats, secrete much more antidiuretic hormone under these conditions. The amounts present after simple decapitation (none in 8 and 0.05–0.2 m.u. per ml. serum in 12) may either represent a higher basal rate of secretion of the hormone or reflect excitement in rodents which were trapped in the Arizona desert a short time before. In terms of concentration in serum, kangaroo rats can secrete much larger quantities of antidiuretic hormone than laboratory rats of the Long-Evans strain (Tables 1, 2 and 3). This finding is consistent with previous work (Ames and van Dyke, 1950) in which it was reported that the small kangaroo rat, weighing about 40 gms., had as much (or more) antidiuretic hormone in its posterior lobe as the adult Long-Evans rat and in about three times the concentration. Urine of kangaroo rats contained more antidiuretic hormone than that of any mammal so far investigated (rat, dog and man).

Ethanol probably inhibits or suppresses the secretion of antidiuretic hormone (see references in van Dyke and Ames, 1951). It is highly probable that the rate of secretion of the hormone is lower if ethanol has been administered before heart puncture (groups G and H compared with Groups C and D Table 3).

It is impossible to state whether adrenalectomy facilitates the secretion of antidiuretic hormone. Possibly the basal rate of secretion was higher after adrenalectomy but could not be detected by the method of assay which, nevertheless, is much more sensitive than that of Birnie *et al.* The difference between groups C and D ($P=0.03$) like the difference between the normal groups C and E, although probably real, has little meaning owing to inability to standardize the excitation of the neurohypophysis. In groups E and F, ether anesthesia was used before heart

puncture or decapitation. The titer of antidiuretic activity, in contrast with groups C and D, appeared to be lower in the adrenalectomized group; however, the difference was not statistically significant ($P = 0.3$).

SUMMARY

Antidiuretic hormone could not be detected in the serum of normal tranquil rats by intravenous assay by which 0.020 milli-unit or more can be estimated. It was detected in the serum of only one of 14 tranquil adrenalectomized rats. However, secretion of antidiuretic hormone occurred in either normal or adrenalectomized rats after heart puncture or ether anesthesia or both; a difference between the two groups was not proved.

Antidiuretic hormone was never detected in the serum of hypophysectomized rats after heart puncture or ether anesthesia.

The concentration of hormone, when present, was the same in serum or heparinized plasma. These and other findings suggest that neurohypophysial hormone is present and that antidiuretic activity is not an artifact of blood coagulation.

Central excitation of the neurohypophysis by heart puncture may be reduced by the administration of ethanol.

The highest concentrations of antidiuretic hormone after heart puncture or ether anesthesia, (0.75 to 1.25 m.u. per ml.) were found in the serum of kangaroo rats (*Dipodomys merriami*).

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THE EFFECT OF ANDROGENS ON THE SUCCINOXIDASE SYSTEM OF RAT LIVER

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INTRODUCTION

ESTROGEN inhibition of the succinoxidase system of rat liver and pituitary tissues was shown by McShan & Meyer (1946). The point of attack was apparently the cytochrome oxidase in the homogenate. Similar results were obtained in a study of the malic dehydrogenase system by Erway *et al.* (1947). Again cytochrome oxidase was apparently blocked. Androgens were also tested for possible inhibitory action on rat liver succinoxidase by McShan *et al.* (1947). No effect was noted with concentrations of steroid up to 3×10^{-4} M. Sodium androsterone sulfate, a water soluble steroid, was one of the preparations tested. Gordon and Elliott (1947) also noted inhibition of the succinoxidase system in rat brain using natural and synthetic estrogens, but they found no effect with aqueous suspensions of testosterone. However, inhibition by testosterone of aerobic respiration of rat brain was demonstrated by the latter group (1947) and of liver and striated muscle by Eisenberg *et al.* (1949).

By using soluble androgens it has been possible to demonstrate inhibition of enzyme systems involving a portion of the Krebs cycle in rat liver.

METHODS

Adult white rats of the Slonaker-Wistar strain were used. The animals were killed by decapitation. Liver tissue was removed, weighed, and placed in cold, distilled water in the all glass homogenizer described by Potter and Elvehjem (1936). Sufficient water was used to prepare a 5% homogenate.

For study of the succinoxidase system 0.2 ml. of homogenate was placed in Warburg vessels containing 0.3 ml. of 4×10^{-3} M. $CaCl_2$, 0.3 ml. of 4×10^{-3} M. $AlCl_3$, 0.4 ml. of 10^{-4} M. solution of cytochrome C., 0.3 ml. of 0.5 M. sodium succinate, and 1.0 ml. of 0.1 M. phosphate buffer (pH 7.4) and water to make 3.0 ml. This is the mixture described in Umbreit's text (1949). One tenth ml. of 5% KOH was placed into the center well of each flask.

For the cytochrome oxidase study, the Warburg reaction vessel contained phosphate buffer, $AlCl_3$, KOH, and water as above. The substrate was 0.3 ml. of 0.114 M. sodium

Received for publication November 26, 1951.

TABLE 1. SUCCINATE QO_2 VALUES FOR RAT LIVER IN THE PRESENCE OF SODIUM ANDROSTERONE SULFATE

Homogenate in ml.	Sodium androsterone sulfate. Concentration final molarity	QO_2	% Inhibition
Control			
0.1	—	98.5	—
0.2	—	86.0	—
Average	—	90.0	—
0.2	9×10^{-5}	73	19
0.2	1.35×10^{-4}	66	27
0.2	1.8×10^{-4}	65	28
0.2	2.3×10^{-4}	60	33
0.2	2.7×10^{-4}	34	62
0.2	3.2×10^{-4}	7	78
0.2	3.6×10^{-4}	0	100

ascorbate, and 1 ml. of 2.4×10^{-4} M. cytochrome C was used in each vessel. The $CaCl_2$ and sodium succinate were omitted. The homogenate was diluted to 1% for this system. The above method is taken from Umbreit (1949).

In both systems the concentration of cytochrome C was shown to be adequate by preliminary experiments. The two androgens studied were sodium androsterone sulfate and testosterone-17 β diethyl aminoethyl carbonate hydrochloride in concentrations of 1.7×10^{-5} M. to 3.6×10^{-4} M. Both compounds are completely water soluble in the concentrations used. Calculations of QO_2 values were based on O_2 consumption during the first 3 or 4 ten minute intervals. The usual time interval between sacrifice of the animal and initial manometer readings was 15 to 20 minutes.

TABLE 2. SUCCINATE QO_2 VALUES FOR RAT LIVER IN THE PRESENCE OF "SOLUBLE TESTOSTERONE"

(TESTOSTERONE-17β DIETHYL AMINOETHYL CARBONATE HYDROCHLORIDE)

Homogenate in ml.	"Soluble testosterone" concentration final molarity	QO_2	% Inhibition
Control			
0.1	—	89	—
0.2	—	84	—
Average	—	87	—
0.2	1.7×10^{-5}	63	29
0.2	2.3×10^{-5}	32	63
0.2	3.4×10^{-5}	22	75
0.2	4.3×10^{-5}	11	86
0.2	5.1×10^{-5}	5.4	94
0.2	8.8×10^{-5}	0	100

RESULTS

Inhibition of the succinoxidase system of rat liver was effected by both hormones as shown in Tables 1 and 2. No difference was noted in responses of male and female rats. By comparing the tables it is seen that soluble testosterone is several times more powerful than the androsterone preparation as an inhibitor. Curves of inhibition (Figs. 1 and 2) reflect increasing inhibition with increased amount of inhibitor.

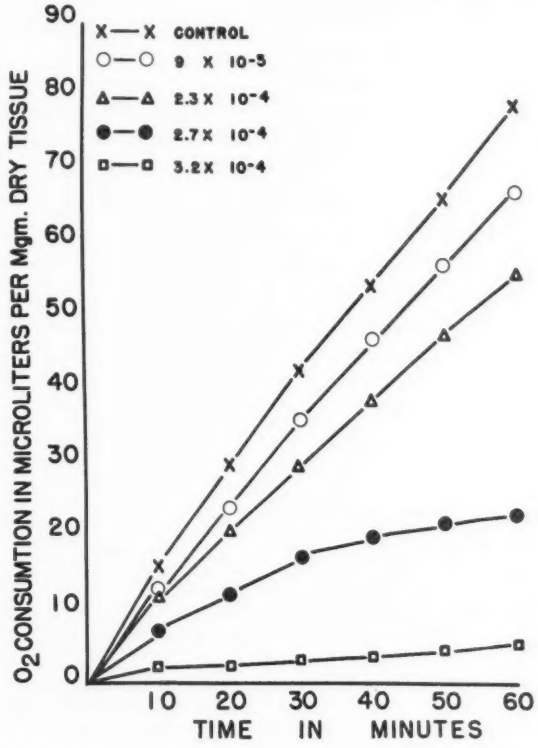

FIG. 1. Inhibition of succinoxidase system by sodium androsterone sulfate. The concentration is expressed in final molarity

In the studies on cytochrome oxidase, using ascorbate as the substrate, concentrations of androsterone which inhibited the succinoxidase system did not affect ascorbate QO_2 values (Table 3). However, higher concentrations of testosterone than were used to inhibit succinate QO_2 were effective inhibitors of ascorbate reduction. An attempt to by-pass the cytochrome oxidase in the succinoxidase system was made by using methylene blue (6×10^{-5} M.) as demonstrated by Grieg (1946). No reversal of inhibition was noted. The addition of cytochrome C and succinate from the side arms of the reaction flasks did not affect the inhibition.

DISCUSSION

Eisenberg *et al.* (1949) postulated that testosterone acts upon the dehydrogenases of the main line of biological oxidation. They based this idea on the following: (1) methylene blue did not reverse the depression of

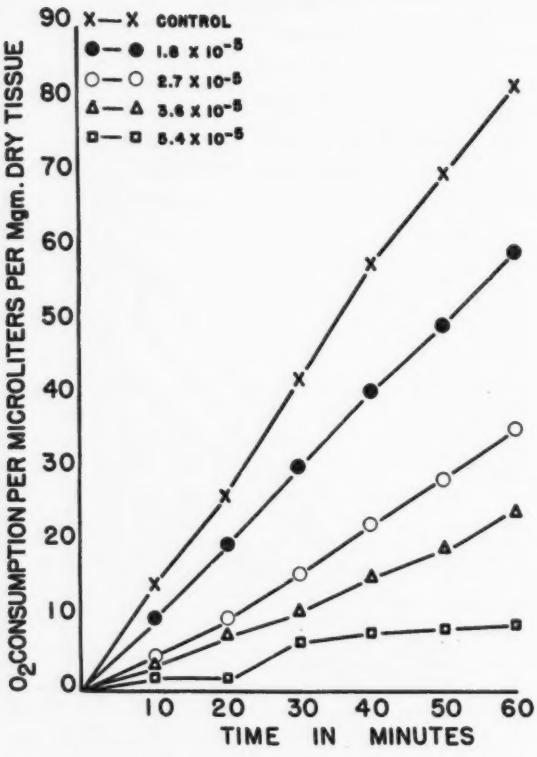

FIG. 2. Inhibition of succinoxidase system by testosterone-17 β diethyl aminoethyl carbonate hydrochloride. The concentration is expressed as final molarity.

TABLE 3. ASCORBATE QO_2 VALUES FOR RAT LIVER IN PRESENCE OF WATER-SOLUBLE ANDROGENS

a. Sodium Androsterone Sulfate
b. "Soluble Testosterone" (Testosterone-17 β diethyl aminoethyl carbonate hydrochloride)

Vessel	Concentration of inhibitor (final molarity)	QO_2	% Inhibition
Control	—	449	—
a. Sodium Androsterone Sulfate			
1	9×10^{-5}	433	(±)
2	9×10^{-5}	460	—
Control	—	446	—
b. "Soluble Testosterone"			
1	3.4×10^{-5}	320	28
2	6.8×10^{-5}	102	77

aerobic respiration effected by testosterone; (2) substitution of pyruvate for glucose as the substrate did not reduce the amount of inhibition; and (3) amounts of testosterone which inhibited glucose oxidation did not inhibit the oxidation of succinate.

Our experiments point also to an inhibition of the dehydrogenase system, in this instance, succinic dehydrogenase. It appears that the cytochrome oxidase in the tissue is not a critical factor although it may be inhibited by fairly high concentrations of hormone. Methylene blue, which is a partial substitute for this enzyme, does not reverse the succinoxidase inhibition, which suggests that the succinic dehydrogenase is affected. Although the testosterone preparation appears to be effective in smaller concentrations than the androsterone the results are not directly comparable because of the different composition of the side chain in each compound. More work is required to establish the exact nature of the inhibition, but it does seem probable that the steroids act directly on the enzyme.

SUMMARY

Inhibition of the succinoxidase system of rat liver is demonstrated for sodium androsterone sulfate and a soluble testosterone preparation (testosterone-17 β diethyl aminoethyl carbonate hydrochloride). The testosterone preparation is the more powerful inhibitor. The inhibition probably involves succinic dehydrogenase. It may involve cytochrome oxidase also, but the latter enzyme appears to be less sensitive to the compounds tested.

Acknowledgements

The author wishes to thank Drs. E. Oppenheimer and A. F. St. André of Ciba Pharmaceuticals, Summit, N. J., for supplying the steroids used in this investigation. He is indebted to Dr. W. C. Cutting for advice during the study and for help in preparing the manuscript.

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CORTICOSTEROIDS OF PERIPHERAL BLOOD[1]

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THE presence of adrenal steroids in peripheral blood has been established by bio-assay (Vogt, 1943; Paschkis *et al.*, 1950) and by chemical determination (Hemphill and Reiss, 1947; Corcoran and Page, 1948; Wick, Hillyard and MacKay, 1951; Nelson, Samuels, Willardson and Tyler, 1951). Adrenal venous blood of the dog contains 17-hydroxycorticosterone in sufficient concentrations to enable isolation and identification (Reich, Nelson, Zaffaroni, 1950). The small amounts of corticosteroid in peripheral blood have discouraged attempts at characterization.[3] However, extracorporeal dialysis by the artificial kidney (Kolff, 1947) and peritoneal lavage (Grollman, Turner and McLean, 1951) techniques have enabled us to obtain, in a partially purified state, a fraction of the total corticosteroids from a relatively large volume of peripheral blood. Paper chromatography of extracts of these dialysates has permitted partial characterization of certain known corticosteroids.

EXPERIMENTAL

As part of another study, two male dogs weighing 20 and 22 kg. respectively were bilaterally nephrectomized. One of these (No. 36–93) was maintained by regular dialysis with the artificial kidney; one such treatment provided nine liters of rinsing fluid. The second dog (No. 38–65) was treated by peritoneal lavage; rinses from four such daily treatments totaled 21 liters. Each batch of dialysate was immediately treated by twice stirring with 1 per cent by weight of activated charcoal (Darco G-60). The charcoal was collected by centrifugation, washed with 1 per cent saline solution and stored in the cold.

Received for publication December 1, 1951.

[1] This investigation was supported (in part) by a research grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

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[3] After completion of this manuscript the study by Nelson and coworkers (1951) on the corticosteroid levels of human blood appeared. By means of paper chromatography 17-hydroxycorticosterone was identified as the major component of the *free* steroids.

Adsorbates were eluted by stirring at room temperature with five 500 ml. volumes each of acetone, benzene and methylene chloride in succession. Each extract was concentrated under reduced pressure, combined with others from the same solvent and evaporated to dryness; the residues were extracted with hot methylene chloride. The total methylene chloride soluble materials from the two adsorbates weighed 327 mg. and 399 mg. respectively. These lots were combined as the non-conjugated steroid fraction.

The two adsorbates were further extracted separately with three 500 ml. volumes of 90 per cent aqueous pyridine (Cook and Grant, 1947; Grant and Beall, 1950). Evaporation of the solvent under reduced pressure and removal of last traces of pyridine in an evacuated desiccator over sulfuric acid yielded a gummy, water-soluble residue.

The residue thus obtained from dog 36–93 was dissolved in 125 ml. of water and allowed to hydrolyze at pH 1.0 for 16 hours in the cold. The aqueous solution was shaken with methylene chloride and the extract evaporated to dryness; the residue weighed 123 mg. After removal of acidic and phenolic components by extraction with 5 per cent sodium bicarbonate and 0.5 N. sodium hydroxide, the neutral fraction weighed 75 mg.; colorimetric determination of reducing steroids (Heard and Sobel, 1946) indicated a content of 6.6 mg. calculated as desoxycorticosterone. No further analysis of this fraction was made.

The product obtained from pyridine elution of the second adsorbate (dog 38–65) was dissolved in 400 ml. of water and adjusted to pH 6.5. Extraction with benzene and methylene chloride yielded fractions containing a total of 576 mg. of material. This was presumed to contain additional non-conjugated steroids and was combined with the free steroid fraction described above. The aqueous fraction (400 ml.) was adjusted to pH 1.0 and shaken five times with 80 ml. of *n*-butanol to separate the conjugated steroids (Cohen and Marrian, 1936). Evaporation of the butanol extract under reduced pressure gave a gummy residue which dissolved in water with the aid of a small volume of dilute alkali. The solution was diluted to 1.5 l., adjusted to pH 4.5 and 100 ml. of 1.0 M. acetate buffer pH 4.5 added; 2.5 g. of calf spleen β-glucuronidase[4] (25,000 units per g.) was stirred in and the mixture incubated at 37° C. for 72 hours in the presence of chloroform. The aqueous solution was then extracted five times with 150 ml. portions of methylene chloride. The extracts were pooled and washed once with water, dried and evaporated. The extractable material released by glucuronidase incubation weighed 244 mg.

Non-conjugated Steroid Fraction: The combined free steroid fractions from both adsorbates, totalling 1302 mg. of methylene chloride-soluble material,

[4] Crude calf spleen β-glucuronidase preparations were obtained from the Viobin Corporation.

were dissolved in 500 ml. of methylene chloride and successively extracted in the cold with five 50 ml. volumes each of 5 per cent sodium bicarbonate and 0.5 N sodium hydroxide. After back extraction with 50 ml. of methylene chloride, the alkaline washes were acidified to pH 1.0 and re-extracted with methylene chloride to obtain acidic and phenolic fractions which weighed 195 and 293 mg. respectively.

The alkali-washed methylene chloride solution was shaken at once with 50 ml. of 0.5 N hydrochloric acid and water. A neutral fraction weighing 342 mg. was obtained after evaporation. Colorimetric determinations indicated contents of 40.4 mg. of reducing steroid and 2.2 mg. of total 17-ketosteroid. An α-ketol reducing steroid estimation of 10–12 mg. was made by evaporating aliquots of the sample on filter paper, exposing the strips to ammoniacal silver reagent (Zaffaroni, Burton and Keutman, 1950) and matching the intensities of the reduced silver areas with those obtained with known amounts of desoxycorticosterone.

The neutral fraction (316 mg.) was dissolved in 300 ml. of hexane and extracted six times with 50 ml. portions of 30 per cent methanol. The combined methanol extracts were extracted three times with 30 ml. portions of hexane; the combined hexane extracts were once more extracted with 30 ml. of 30 per cent methanol. The combined hexane solutions yielded 255 mg. of residue, of which 10.6 mg. were reducing material by the Heard-Sobel test. Chromatography with benzene-formamide showed no trace of α-ketol steroid in this fraction, although there was present considerable amounts of a reducing substance, which appeared as a fast-moving component on chromatogram strips exposed to osmium tetroxide vapors.[5]

The 30 per cent methanol solutions yielded 39.1 mg. of residue. A solution of this residue in 1.0 ml. of methanol, containing approximately 1.2 mg. of α-ketol steroid by the ammoniacal silver test, was subsequently used in such chromatographic characterizations as the small amounts of corticosteroid permitted. Filter paper chromatography in benzene-formamide was done by the method of Zaffaroni, Burton and Keutman (1950) and coworkers (Zaffaroni, Burton and Keutman 1950; Burton, Zaffaroni and Keutman 1951) on 0.1 ml. samples of the methanolic solution. Results are shown in figure 1. Exposure of the paper strip to ammoniacal silver reagent (strip 1) revealed two reducing (α-ketol) steroids (I and II) in the $C_{21}O_5$ zone and two (III and IV) in the $C_{21}O_4$ zone. A duplicate (strip 2) exposed to osmic acid vapor darkened only in the area corresponding

[5] The 1 per cent aqueous osmic acid reagent described by Manaro and Zygmuntowicz (1951) proved to be an equally good and much more sensitive (minimum amount of steroid 20 μg.) reagent for α,β-unsaturated ketosteroids than aqueous 2,4-dinitrophenylhydrazine (minimum 100 mg.).

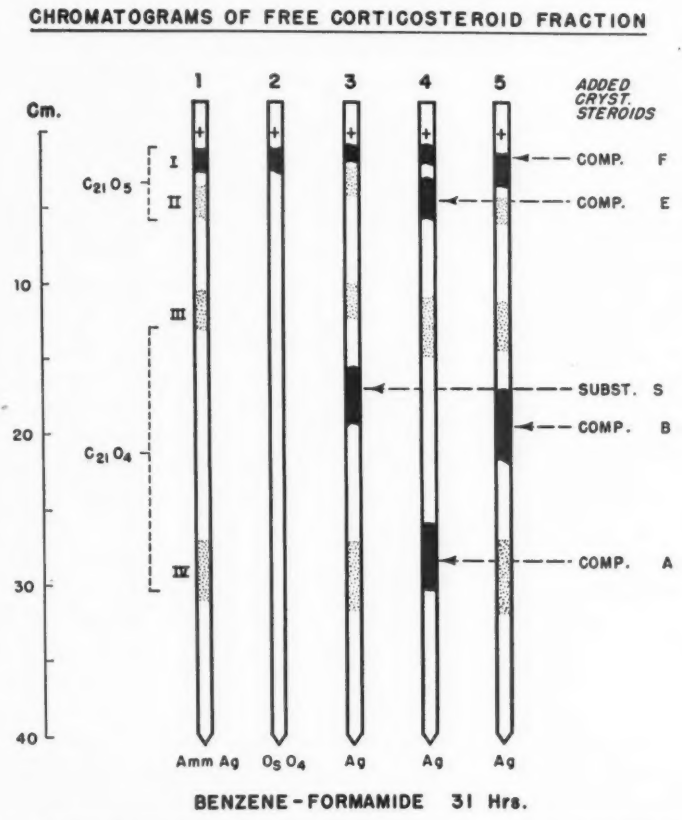

FIG. 1. Diagrams of chromatographic separation of free corticosteroid fraction (3.9 mg. per strip). Comp. A = 11-dehydrocorticosterone; Comp. B = corticosterone; Subst. S = 17-hydroxy-11-desoxycorticosterone; Comp. E = 17-hydroxy-11-dehydrocorticosterone; Comp. F = 17-hydroxycorticosterone.

to reducing steroid I, indicating the presence of an α,β-unsaturated ketone. Corticosteroids I and II did not react with iodine-potassium iodide reagent to give the blue color characteristic of 17-hydroxy-11-dehydrocorticosterone. A strip (not shown) treated with sulfuric acid showed in ultraviolet light the characteristic green fluorescence of the 11-hydroxyl group at the level of steroid I. Replicate strips were chromatographed after addition of solutions of pure corticosteroids; 17-hydroxy-11-desoxycorticosterone was added to strip 3; 17-hydroxy-11-dehydrocorticosterone and 11-dehydrocorticosterone to strip 4; 17-hydroxycorticosterone and corticosterone to strip 5. The coincidence of 17-hydroxycorticosterone with

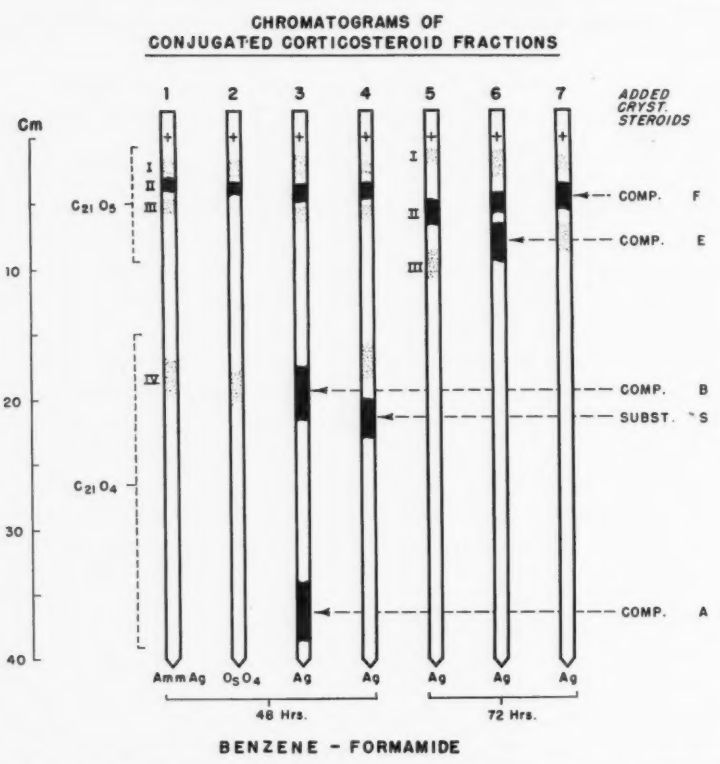

FIG. 2. Diagrams of chromatographic separation of conjugated corticosteroid fraction (3.04 mg. per strip). Comp. A = 11-dehydrocorticosterone; Comp. B = corticosterone; Subst. S = 17-hydroxy-11-desoxycorticosterone; Comp. E = 17-hydroxy-11-dehydrocorticosterone; Comp. F = 17-hydroxycorticosterone. Strip 4 shows an anomalous separation of Comp. B and Subst. S in benzene-formamide.

steroid I (strip 5), together with the evidence for the α,β-unsaturated ketone and 11-hydroxy functions provides identification of steroid I as 17-hydroxycorticosterone.

The faint reduction found in strip I at the level of steroid II was intensified by addition of 17-hydroxy-11-dehydrocorticosterone (strip 4). However steroid II showed no α,β-unsaturated ketone function by the osmic acid reaction and did not give a positive reaction with iodine-potassium iodide reagent, thereby establishing the absence of the 11-keto compound from the extract. Steroids III and IV, possessing the α-ketol function and lacking the α-β-unsaturated ketone structure were not further characterized. The chromatographic mobility of these compounds with respect to

the $C_{21}O_4$ group would suggest a possible structure having a partially or totally saturated ring A, similar to the "tetrahydro E and F" type of α-ketol steroids recently found in urine (Schneider 1950, Kinsella *et al.*, 1951, Lieberman *et al.*, 1951).

Conjugated steroid fraction: The material released by β-glucuronidase was dissolved in 250 ml. of methylene chloride. The solution was washed with five 50 ml. portions of 0.5 N sodium hydroxide in the cold; the combined alkaline extracts were extracted twice with 25 ml. of methylene chloride. The combined methylene chloride solutions were washed once with 25 ml. portions of 0.5 N hydrochloric acid and water, dried, and evaporated to dryness. The residue weighed 118 mg. and was partitioned between hexane and 30 per cent aqueous methanol. The hexane-soluble fraction weighed 88 mg. The aqueous methanol after evaporation and extraction with methylene chloride yielded 30.4 mg. This residue was dissolved in 1.0 ml. of methanol and the corticosteroid concentration estimated as 0.3 to 0.5 mg. by the ammoniacal silver spot test.

Paper chromatographic separations were made in benzene-formamide solvent (figure 2) using 0.1 ml. of the methanol solution for each filter paper strip. Visualization of the steroids on the strip with ammoniacal silver (strip 1) revealed four distinct areas. The relative concentrations of each per 0.1 ml. of solution were approximately 5 μg. of the single $C_{21}O_4$ steroid (IV), and approximately 2 μg. of (III), 20 μg. of (II), and 15 μg. of (I) for the three $C_{21}O_5$ steroids, steroid III failed to react with the osmic acid reagent (strip 2).

The $C_{21}O_4$ steroid (IV) was characterized by means of mixed chromatograms with added crystalline corticosterone and 11-dehydrocorticosterone (strip 3) and 17-hydroxy-11-desoxycorticosterone (strip 4) in which steroid IV migrated at a rate similar to that of corticosterone and different from that of the other two. Sulfuric acid added to the region of steroid IV produced the typical green fluorescence in ultaviolet light. Of the three $C_{21}O_5$ steroids (I, II, III), only steroid II was characterized by means of mixed chromatograms. Crystalline 17-hydroxycorticosterone added to the extract (strip 7) moved to a position coincident with $C_{21}O_5$ steroid II, while added 17-hydroxy-11-dehydrocorticosterone migrated more rapidly (strip 6) than steroid II. The sulfuric acid-fluorescence test was positive at the site of steroid II, thereby confirming its identity as 17-hydroxycorticosterone.

Although similar in mobility to steroid III, 17-hydroxy-11-dehydrocorticosterone (strip 6) appeared to be absent from the corticosteroid extract by the failure of the iodine-potassium iodide reagent to produce the blue coloration typical of the latter steroid. Steroid III was therefore character-

ized only as a $C_{21}O_5$ steroid possessing an α-ketol grouping and lacking the α,β-unsaturated ketone structure. Steroid I, possessing both these features was not further characterized.

DISCUSSION

Complete chemical characterization of the corticosteroids found in the blood dialysates was not feasible in view of the very small amounts (1.2 mg. of non-conjugated and 0.4 mg. of conjugated material) available for this study. However, relative chromatographic mobilities, chemical tests and comparisons of mixed chromatograms with added crystalline steroids indicate that the principal corticosteroid in both conjugated and free fractions was 17-hydroxycorticosterone. It was also possible to recognize corticosterone in low concentration as a component of the conjugated steroid fraction. The presence of these two steroids and the absence of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone agree with studies (Reich, Nelson and Zaffaroni, 1950) of the corticosteroids of dog adrenal venous blood.[6] Also present in peripheral blood are steroids apparently possessing the α-ketol side chain but lacking the α,β-unsaturated ketone grouping. Their properties resemble those of the so-called "tetrahydro E and tetrahydro F" compounds recently found in urine (Schneider, 1950; Lieberman *et al.*, 1951), but would appear to be of a lower degree of oxygenation, possibly of the $C_{21}O_4$ group. The data do not give any quantitative evaluation of the corticosteroid concentrations of peripheral blood since neither the volume of blood exposed to dialysis nor the completeness of dialysis can be evaluated. The fact that the blood contains corticosteroids conjugated with glucuronic acid in addition to those directly extractable by lipid solvents is in keeping with the current notion that most, if not all urinary corticosteroids are excreted as glucuronides (Lieberman and Dobriner, 1951). It also agrees with studies on blood estrogen, up to one-half of which in serum occurs in the conjugated state (Rakoff *et al.*, 1934; Szego and Roberts, 1946). Certain corticosteroids (Lewis and Savard, unpublished data) like the estrogens (Roberts and Szego, 1946), have been found to be associated with the β-lipoprotein fraction of serum. The possibility of the conjugated corticosteroids being "protein-bound" is recognized but as yet undetermined. Tacit recognition of the obscurity of these chemical associations is the bio-assay procedure (Paschkis *et al.*, 1950) which foregoes chemical extraction of corticosteroid and utilizes diluted but otherwise intact serum.

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[6] The occurrence of corticosterone and 17-hydroxycorticosterone in dog's adrenal venous blood has been verified in these laboratories (Savard and Masson, Unpublished data), a ratio of 1:2 between the two corticosteroids, however, being observed.

SUMMARY

Dialysates obtained from dogs by the use of the artificial kidney and peritoneal lavage were examined for the presence of adrenal corticosteroids. These were found to be present in a free, lipide-soluble state and in a water-soluble, lipide-insoluble state. The corticosteroids present in the latter form were rendered lipide-soluble by incubation with β-glucuronidase and may be presumed to have existed as glucuronides. The principal corticosteroid of both fractions, identified by paper chromatography, was found to be 17-hydroxycorticosterone; corticosterone was detected only in the conjugated fraction. Several other steroids which possessed reducing groups but lacked the α,β-unsaturated ketone grouping were detected but not identified.

Acknowledgements

The technical assistance of Ruth Wright and Mary Ann Dvorak is acknowledged. Dr. Max Tishler, of Merck and Company generously supplied crystalline 17-hydroxy-11-dehydrocorticosterone, and the acetates of 11-dehydrocorticosterone and 17-hydroxycorticosterone from which the free compounds were prepared. Corticosterone was supplied by Dr. Ralph Dorfman, and 17-hydroxy-11-desoxycorticosterone, as the acetate, through the courtesy of Mr. Adrian D. Joyce of the Glidden Company.

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NOTES AND COMMENTS

THE ABSORPTION OF THYROXINE FROM THE GASTROINTESTINAL TRACT OF THE RAT

In a previous study (Johnson and Albert, 1951) a considerable accumulation of I^{131} was observed in the gastrointestinal tract of the rat after subcutaneous administration of either inorganic radioiodide or radiothyroxine. During the first six hours following injection of NaI^{131}, the stomach contained twice as much radioactivity per gram wet weight as did the thyroid. Similarly, after administration of radiothyroxine, considerable I^{131} was present in the stomach, and in the small bowel. As the concentration in the small bowel decreased during the first six hours, the radioactivity in the large bowel rose. These findings, plus the added information that radiothyroxine was also concentrated in the liver (Gross and Leblond, 1947; Johnson and Albert, 1951), suggest that the gastrointestinal tract may play a significant role in the metabolism of thyroid hormone.

The content of radiothyroxine or of its metabolites in any portion of the gastrointestinal tract at any interval after subcutaneous injection may depend on a number of factors: secretion into the bowel, absorption from the bowel, transport of material from one to another portion of the bowel, delivery to the bowel by way of the biliary system. It seemed desirable to study each of these factors separately. Accordingly the present experiments were performed to determine the absorption of thyroxine from, and secretion into, the three major portions of the gastrointestinal tract. As a control study, the behavior of inorganic radioiodide was simultaneously observed.

MATERIAL AND METHODS

Male rats of the Wistar strain, ranging from 50 to 100 gm. in weight, were used. With the rats under ether anesthesia the stomachs of 13 animals were ligated at both pyloric and esophageal poles. In 7 animals, radioiodide was injected into the lumen of the stomach, thus isolated from the remainder of the bowel. In the remaining 6 animals, radiothyroxine was similarly injected. The dose of radioiodide was 5 microcuries containing 5 micrograms stable NaI; that of radioactive l-thyroxine was 5 microcuries with a specific activity varying from 1 to 17 millicuries per milligram (Abbott Laboratories, North Chicago, Illinois). Some of the rats were killed at two hours, and some at five hours after injection. One cubic centimeter of blood was withdrawn, and the stomach, small bowel, large bowel, and liver were removed. The amount of radioactivity in each of these organs was determined (Johnson and Albert, 1951), and expressed as percentage of the dose injected. Similarly, in 12 animals, the small bowel was isolated by placing ligatures at the pyloric end and at its junction with the large bowel. In 6 of the rats radioactive thyroxine and in 6 others radioiodide was injected into the lumen of the small bowel and the analytic procedures described were repeated. Finally in 12 animals the large bowel was isolated by ligatures near the anus and at its junction with the small bowel. The two radioactive substances were injected into the lumen of the isolated large bowel and the same procedures were carried out.

RESULTS

Table 1 shows that very little inorganic iodide was absorbed from the stomach. However, the major portion of NaI^{131} placed in either the small or the large bowel was absorbed and resecreted into the stomach. This is confirmatory of our previous studies which showed that the stomach comprised a considerable portion of the total iodide space. Under normal physiologic conditions, the iodide secreted into the stomach from the blood is probably passed along into the small bowel, from which it is reabsorbed into the blood.

Received for publication January 7, 1952.

TABLE 1. I^{131} IN VARIOUS ORGANS AFTER INJECTION OF RADIOIODIDE AND RADIOTHYROXINE INTO THREE MAJOR SITES OF THE GASTROINTESTINAL TRACT

Material and site of injection	Animals	Time, hr.	Percentage of dose administered (mean values)				
			Stomach	Small bowel	Large bowel	Liver	Blood
Radioiodide							
Stomach	5	2	96.22	0.23	0.09	0.16	0.10
	2	5	91.54	0.76	0.47	0.29	0.06
Small bowel	4	2	63.36	4.67	0.97	0.92	0.70
	2	5	77.63	1.88	0.24	0.27	0.13
Large bowel	4	2	32.67	7.34	3.68	2.10	1.36
	2	5	29.15	4.08	2.39	2.10	0.96
Radiothyroxine							
Stomach	4	2	92.78	0.32	0.02	0.20	0.00
	2	5	95.27	0.56	0.01	0.18	0.01
Small bowel	4	2	4.19	81.12	0.28	1.05	0.26
	2	5	8.23	78.57	0.43	3.51	0.38
Large bowel	4	2	2.63	3.47	76.53	1.09	0.20
	2	5	6.95	6.10	76.58	1.39	0.35

Similarly, little thyroxine was absorbed from the stomach, as indicated by the negligible concentration of I^{131} in the blood. However, 20 to 25 per cent of the radioactive thyroxine placed in either the small or the large bowel was removed. Part of the radioactive material absorbed from the isolated small or large bowel was resecreted into the stomach.

DISCUSSION

Large quantities of inorganic iodide are secreted into the stomach and reabsorbed by the small and the large bowel. This enteric circulation of iodide involves a significant portion of the total iodide of the body, but is obviously in equilibrium with, and dependent on, the systemic circulation of iodide. The absorption of thyroxine from the intestinal tract is much less efficient, and in view of the high fecal excretion noted by Johnson and Albert, the enteric circulation of thyroxine is not in similar equilibrium with that of the blood. Hence, a significant amount of thyroxine may be lost to the body under normal conditions via the feces.

What proportion of the radioactivity found in the bowel after subcutaneous injection of radiothyroxine reaches the gut by direct secretion from blood, or by secretion through the biliary system remains to be determined. In any case, the enteric or possibly enterohepatic circulation of thyroid hormone appears to be of considerable significance in normal physiologic conditions, since the results reported by Johnson and Albert with labeled l-thyroxine should apply with equal force to endogenously produced thyroxine. Also of importance is that the existence of an enterohepatic circulation of thyroxine presents opportunity for chemical transformation of thyroid hormone, such as was reported by Taurog, Briggs and Chaikoff (1951), changes which may be of physiologic significance in the intermediary metabolism of thyroidal substances.

SUMMARY

Insignificant amounts of inorganic iodide or thyroxine were absorbed from the iso-

lated stomach within five hours after intragastric injection. Virtually all of the iodide, and 20 to 25 per cent of the thyroxine, were absorbed from the isolated small or large bowel within five hours after injection into the lumen of these organs.

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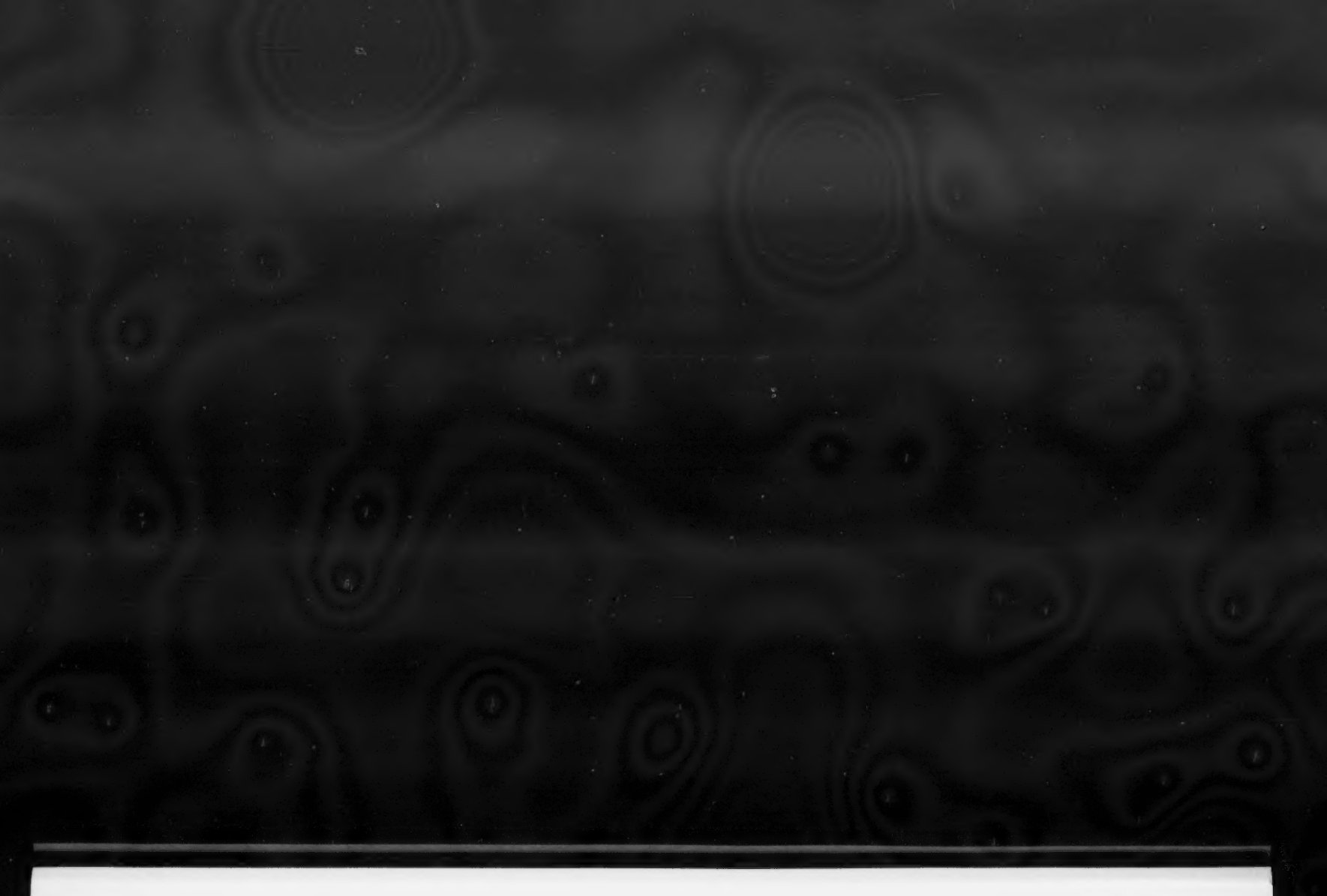
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